Identifying Key Regulators of the Immune/Inflammatory Response for Developing Anticonvulsive and Antiepileptogenic Approaches

Thesis

IDENTIFYING KEY REGULATORS OF THE IMMUNE/INFLAMMATORY RESPONSE FOR DEVELOPING ANTICONVULSIVE AND ANTIEPILEPTOGENIC APPROACHES

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La miniera è esattamente là dove si è: basta scavare.
Tiziano Terzani

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
AD	Alzheimer's disease
ADT	Afterdischarge threshold
ALXR	Lipoxin A_4 receptor
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP-1	Activator protein-1
AQP4	Aquaporin4
ATF3	Activating transcription factor 3
ATL	Aspirin-triggered lipoxin A_4
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood brain barrier
BNDF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CA	Cornu ammonis
CBZ	Carbamazepine
CD11b	Integrin alpha M
ChemR23	Chemerin receptor 23
COX-2	Cyclooxygenase-2
CNS	Central nervous system
CREB	cAMP responsive element binding
csf	Cerebrospinal fluid
CXCL12	C-X-C motif chemokine ligand 12
CX3CL1	fractalkine

CX3CR1	fractalkine receptor
CXCR4	C-X-C motif chemokine receptor 4
CCL2	C-C chemokine ligand 2
CCLR2	C-C chemokine receptor 2
CyP	Cyanobacterial LPS
DAB	Diaminobenzidine
DAMP	Damage associated molecular pattern
DHA	Docosahexaenoic acid
DPA	Docosapentanoic acid
EEG	Electroencephalographic
ELISA	Enzyme-immunosorbent assay
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
FCD	Focal cortical displasia
FIRES	Febrile infection-related epilepsy syndrome
FPR	Formyl peptide receptor
GABA	Gamma-aminobutyric acid
GAERS	Genetic absence epilepsy rat from Strasbourg
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter-1
GluR	Glutamate receptor
GPCR	G-protein coupled receptor
HCN	Hyperpolarization-activated cyclic nucleotide-gated potassium channel
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
KA	Kainic acid
Kir	Inward rectifying potassium channels

ICE/caspase-1	Interleukin-1 converting enzyme
ICV	Intracerebroventricular
IFN-γ	Interferon-γ
ILAE	International league against epilepsy
IL-1β	Interleukin-1 beta
IL-1Ra	Interleukin-1 receptor antagonist
iNOS	Inducible form of nitric oxide synthase
IRAK	Interleukin 1 receptor associated kinase 1
LC/MS-MS	Liquid chromatography mass spectrometry
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LX	Lipoxin
MAGL	Monoacylglycerol lipase
MAPK	Mitogen activated protein kinase
miRNA	microRNA
NeuN	Neuronal nuclei
NF-κB	Nuclear factor-kappa B
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NORT	Novel object recognition test
NPY	Neuropeptide Y
NRSF/REST	Neuron-restrictive silencer factor
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD1	Protectin D1
PG	Prostaglandin

PLA2	Phospholipase A2
PN	Postnatal
PNS	Peripheral nervous system
PUFA	Polyunsaturated fatty acid
RAGE	Receptor for advanced glycation end product
ROC	Receiver operating characteritics
ROS	Reactive oxygen species
RT-qPCR	Real time polymerase chain reaction
Rv	Resolvin
S100β	S100 calcium-binding protein β
SE	Status epilepticus
SPM	Specialized pro-resolving mediator
SUDEP	Sudden unexplained death in epilepsy
TBI	Traumatic brain injury
TBS	Tris-HCl-buffered saline
TGF-β	Transforming growth factor-β
TIR	Toll/Interleukin-1 receptor
TLE	Temporal lobe epilepsy
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α
TrkB	Tyrosine kinase receptor B
TSC	Tubero sclerosis
VPA	Valproic acid

ABSTRACT

Epilepsy therapy is based on drugs that treat the symptoms, seizures, rather than the disease. There are no treatments for modifying the course of the disease, improving prognosis. Another unmet need is the absence of biomarkers able to identify patients at risk of developing epilepsy or with a progressive disease. Among the potential pathways for attaining these unmet needs, we focused on neuroinflammation since it is a pathological process occurring in experimental epileptogenesis and in human epilepsy. Interleukin-1β (IL-1β)/IL-1Receptor type 1 (IL-1R1) and High Mobility Group Box 1 (HMGB1)/Toll-like Receptor 4 (TLR4) axes are key initiators of neuroinflammation following epileptogenic injuries. Experimental and clinical evidence showed that endogenous anti-inflammatory mechanisms are not efficiently activated in epileptogenic tissue. Among these mechanisms, we focused on specialized mediators driving the process of "resolution of inflammation".

We demonstrated that induction of pro-resolving response is delayed during epileptogenesis compared to activation of neuroinflammation. Based on this, we studied the antiepileptogenic and disease-modifying effects of two pharmacological strategies aimed at (1) anticipating and boosting resolution of neuroinflammation by enhancing pro-resolving mechanisms and (2) blocking the neuroinflammatory response with a combined anti-inflammatory treatment targeting IL-1β/IL-1R1 and HMGB1/TLR4 pathways. (1) The pro-resolving treatment decreased neuroinflammation, ameliorated cognitive deficits and reduced spontaneous seizure frequency and duration; (2) the combined anti-inflammatory treatment prevented disease progression.

The role of HMGB1 and its isoforms was investigated in epileptogenesis and drug-resistance. In two animal models of epileptogenesis the pathologic disulfide HMGB1 isoform progressively increased in blood before epilepsy onset, and prospectively identified animals that developed the disease. Moreover, the combined anti-inflammatory approach prevented disease progression and the increase in HMGB1 isoforms in blood during epileptogenesis.

In conclusion, pro-resolving and anti-inflammatory treatments have disease-modifying effects and HMGB1 isoforms are potential mechanistic biomarkers for epileptogenesis, underlying the need of validation in prospective clinical studies.

CHAPTER 1 - INTRODUCTION

1.1 EPILEPSY

1.1.1 Definition and classification

Epilepsy is a neurological disorder characterized by a persistent predisposition of the brain to develop epileptic seizures and the resulting neurobiological, cognitive, psychological and social consequences (Fisher et al., 2014). The term seizure refers to "the transient occurrence of signs and/or symptoms due to abnormal, excessive or synchronized neuronal activity, detectable by electroencephalographic analysis (EEG)". This phenomenon can be localized in a specific area (focal onset seizure), or spreading to neuronally connected brain regions (focal to bilateral onset seizure) or may involve both hemispheres (generalized onset seizure) (Fisher, 2017). The epileptic seizure can be associated with convulsive, sensitive, emotional and neurovegetative manifestations.

From a clinical perspective, epilepsies can be divided into three main groups (Trinka et al., 2015). The first group include genetically determined epilepsies, they are the direct consequences of one or more genetic defects, known or suspected.

The second group refers to epilepsies associated with various structural or metabolic conditions which can be acquired (e.g., neurotrauma, *status epilepticus*, stroke, infection, febrile seizures) or genetic (e.g., tuberous sclerosis, malformations of cortical development). These phenomena increase the risk of developing epilepsy, triggering the epileptogenesis process, which will be described later in Section 1.2. Typically, following a brain insult, there is a prodromal phase lasting from days to years before the onset of spontaneous recurring epileptic seizures (Loscher et al., 2013). Of particular relevance among acquired epilepsies is Temporal Lobe Epilepsy (TLE), which represents the most common form of human epilepsy characterized by drug-refractory focal onset seizures. The most frequent epileptic syndrome of temporal lobe-origin is Mesial Temporal-Lobe Epilepsy, characterized by hippocampal sclerosis. One of the potential causes of TLE and other acquired epilepsies most often mimicked in animal models is *status epilepticus* (SE), "a clinical situation in which a seizure is manifested

continuously for more than 5 minutes, or in which multiple seizures are repeated at very short intervals representing a continuous condition of epileptic activity" (Trinka et al., 2015).

The third group includes epilepsies of unknown etiology in which the cause underlying seizures is unknown, although it is probably on a genetic basis; alternatively, epilepsy may be the result of a structural or metabolic disorder that has not been diagnosed yet (Berg and Scheffer, 2011).

1.1.2 Unmet clinical needs

Epilepsy is one of the most common diseases of the central nervous system (CNS)(Klitgaard, 2013); it affects about 70 million people worldwide with an estimated incidence of 34 to 76 new cases per year per 100,000 people (Abraham and Shaju, 2013; Loscher et al., 2013; Schmidt and Schachter, 2014). In low- and middle-income countries, estimates of epilepsy's prevalence are generally higher (Schmidt and Schachter, 2014). In Europe, it has been estimated 6 million patients with active epilepsy, and the annual health cost associated with epilepsy is over 20€ billion (Schmidt and Schachter, 2014). Epileptic seizures reduce the quality of life of patients, increase the risk of death, injury, and impose socioeconomic and educational disadvantages. Neurological comorbidities are associated with epilepsy, including cognitive deficits, depression, anxiety, and high suicide rates (Loscher et al., 2013; Schmidt and Schachter, 2014). Uncontrolled seizures and the progression of epilepsy can compromise memory, cognition, and endocrine function (Loscher et al., 2013). Mortality in patients with epilepsy, including sudden unexplained death (SUDEP), is three times the rate observed in the general population (Loscher et al., 2013; Schmidt and Schachter, 2014).

Despite the availability of a significant number of anti-seizure drugs, it is estimated that around 30% of newly diagnosed epilepsy patients remains resistant to therapies (Abraham and Shaju, 2013; Loscher et al., 2013). Traditionally, drug resistance is defined as the failure of seizures to respond to at least two anti-seizure drugs that are appropriately chosen, adequately dosed, and used for an appropriate period (Schmidt and Schachter, 2014).

The incidence, the costs, the increased risk of death, the social burden and the absence of effective treatment for some patients indicate that there is an urgent need to find new strategies to prevent the onset of the disease in individuals at risk or for improving the clinical course of the disease in those patients who have a poor prognosis (Loscher et al., 2013).

1.2 EPILEPTOGENESIS

1.2.1 Definition

Epileptogenesis is a dynamic process by which a brain network, previously normal, is functionally altered toward a decreased seizure threshold, thus having an increased probability to generate spontaneous seizures (Dudek and Staley, 2012).

Epileptogenesis can be triggered by genetic or acquired factors and can continue after epilepsy diagnosis.

Traditionally, epileptogenesis has been considered a latent period between the precipitating insult and the appearance of the first clinical seizure. Many studies provided evidence that human epilepsy may have a progressive course in a significant percentage of cases (Pitkanen and Sutula, 2002; Schmidt and Sillanpaa, 2012), thus suggesting that epileptogenesis is a continuous and prolonged process. Furthermore, animal models have shown that various molecular, cellular and functional modifications, which are proposed to lead to the occurrence of the first unprovoked seizure, also persist after the onset of seizures and may contribute to the progression of the epileptic condition (Bertram and Cornett, 1993, 1994; Dudek and Staley, 2012; Hellier et al., 1998; Nissinen et al., 2000; Pitkanen and Lukasiuk, 2011; Pitkanen and Sutula, 2002; Schmidt and Sillanpaa, 2012; Williams et al., 2009). Based on this conceptual evolution, the ILAE revised the definition of epileptogenesis. The major difference is that epileptogenesis no longer refers only to the time period between the epileptogenic insult and the diagnosis of epilepsy (Figure 1.2.1A); rather, it includes the mechanisms of progression of the pathology that can continue to occur even after the diagnosis of epilepsy (Figure 1.2.1B). These

conceptual changes have important implications for studying animal models of epileptogenesis, in particular, for the development and testing of potential treatments, and for biomarkers identification (Pitkanen and Engel, 2014; Pitkanen et al., 2013, 2015).

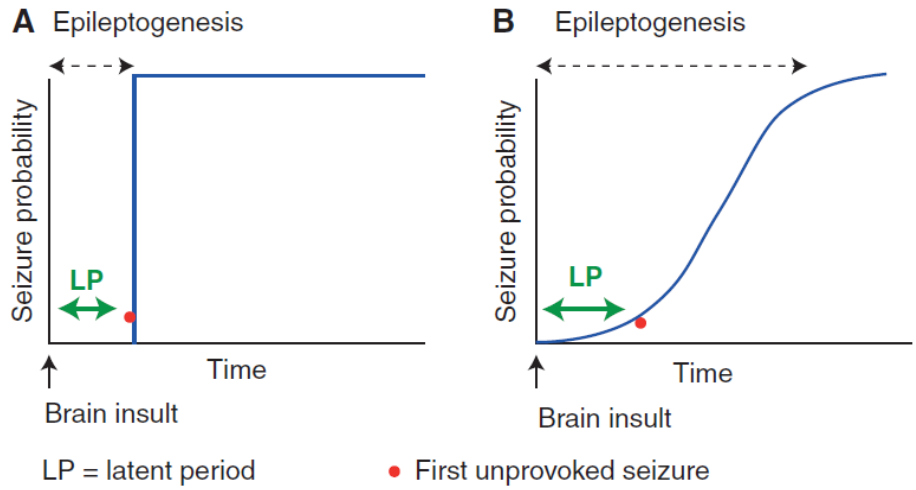


Figure 1.2.1. Definitions of epileptogenesis. *(A) Epileptogenesis was considered to be represented by the latent period between the precipitating insult and the occurrence of the first unprovoked clinical seizure. (B) Recently, based on experimental and clinical observations, epileptogenesis is considered to extend beyond the latent period (Pitkanen et al., 2015).*

Epileptogenesis is often associated with comorbidities such as anxiety, depression and cognitive deficits which may originate from networks overlapping with those causing seizures (Kanner et al., 2014) or result from the effects of spontaneous recurrent seizures. There is intense research focused on studying the mechanisms underlying comorbidities. Notably, animal models showed that cognitive deficits arise before epilepsy onset, thus indicating that comorbidities are not merely a consequence of seizures (Kobow et al., 2012; Pascente et al., 2016).

Anti-epileptogenesis interventions could be designed not only for prevention of disease development (antiepileptogenic), but also for providing disease-modification such as reducing the frequency, duration or severity of seizures, changing seizures from drug-resistant to drug-responsive and/or improving the related pathological outcomes. Comorbidity-modifying treatments may alleviate or reverse anxiety, depression, somatomotor impairment, or

cognitive decline (Pitkanen and Engel, 2014; Pitkanen et al., 2013, 2015). The development of effective therapies to prevent or treat epileptogenesis is an urgent unmet clinical need since the available therapies are mostly symptomatic and anti-ictogenic, aimed at suppressing or reducing the established seizures (Figure 1.2.2).

The design of clinical trials for novel antiepileptogenic and disease-modifying therapies demands the identification of non-invasive biomarkers that allows the identification of patients at high risk of developing epilepsy or patients with a progressive disease, as well as the prediction of comorbidities and pharmacoresistance (Figure 1.2.2) (Terrone et al., 2016) and possibly predicting the therapeutic response to new investigational drugs.

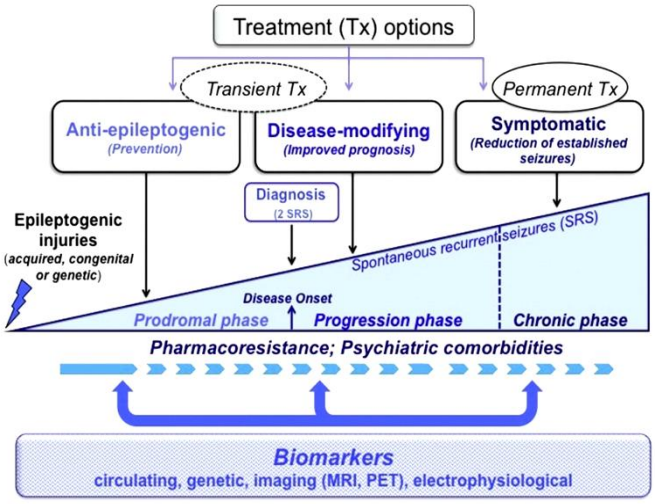


Figure 1.2.2. Epileptogenesis, treatment options and the need of biomarkers. *A therapeutic antiepileptogenic agent must successfully intervene in the epileptogenic process, preventing the development of seizures. A disease-modifying drug should alter the course of the disease resulting in an improved prognosis. Currently available anti-seizures drugs are symptomatic and anti-ictogenic, designed for the reduction of established seizures. The discovery of circulating, genetic, imaging or electrophysiological biomarkers of epileptogenesis and treatment response would greatly facilitate the design of pre-clinical and clinical trials (Terrone et al., 2016).*

1.2.2 Identification of biomarkers of epileptogenesis

Nowadays, there are no sensitive and specific biomarkers to identify individuals at risk of developing epilepsy. In 2013, the ILAE published a taskforce report (Engel et al., 2013) in which a biomarker is defined as "an objectively measurable characteristic of a normal or pathologic process. Biomarkers of epileptogenesis could (1) predict the development of epilepsy, (2) identify the presence of tissue capable of generating seizures, (3) measure the progression after the pathology is established, (4) be used to create animal models for more cost-effective screening of potential antiepileptogenic drugs and devices, and (5) reduce the cost of clinical trials of potential antiepileptogenic therapies by increasing the number of patients at high risk of developing epilepsy to enroll in the clinical studies". An ideal biomarker should be sensitive, specific and easily accessible. In this sense, possible candidates include circulating molecules, genetic markers, non-invasive molecular brain imaging or electrophysiological recordings.

Given the complexity of epilepsy, it is unlikely that a single biomarker will be sufficient for predicting epileptogenesis; a combined approach may be necessary to identify appropriate biomarkers at different stages of the evolution of the disease (Loscher et al., 2013). To this end, a number of alterations in gene expression have been identified in models of acquired epilepsy underlying various molecular and cellular processes linked to epileptogenesis, such as cell death, neurogenesis, neuronal plasticity, immune responses. These alterations develop in a time-specific manner after the primary insult (Loscher et al., 2013; Pavlov and Schorge, 2014). For this reason, it is important to understand the time-dependent evolution of the different molecular and cellular changes occuring during epileptogenesis in order to set up a rational panel of biomarkers.

1.2.3 Potential mechanisms underlying epileptogenesis

An intensive research effort has been focusing on understanding the mechanisms underlying epileptogenesis. Today, our knowledge of the epileptogenic process is largely based on studies in rats and mice in which epileptogenesis is induced by structural alterations of the brain tissue, infections, fever or it evolves as a consequence of gene mutations (Table 1.2.1). The most often experimentally used brain insults, mimicking those in humans, are SE, traumatic brain injury (TBI) and stroke. Most of the currently available molecular and cellular data on the epileptogenic process originate from models in which epileptogenesis is initiated by SE in adult rodents (Table 1.2.2) (Pitkanen and Lukasiuk, 2009).

Table 1.2.1. *Epileptogenesis models.*

ETIOLOGIES	RODENT MODELS
STRUCTURAL	
Neurotrauma*	Fluid percussion; Controlled cortical impact; Cortical undercut
De novo status epilepticus*	Electrical stimulations; Chemoconvulsants; Hyperthermia
Unilateral hippocampal sclerosis*	Intra-hippocampal/cortical kainic acid; Perforant path stimulation
Stroke*	Cortical phototrombosis; Permanent middle cerebral artery occlusion; Endothelin-1 (hippocampal lesion in rats)
Blood-brain barrier damage*	Sub-chronic albumin or TGF-β1 intracerebroventricular infusion
Developmental epileptic encephalopathies	Hypoxia-ischemia injury in rats; *Infantile spasms*: Multiple hit rat model; Tetrodotoxin in rats
Cortical dysplasia	*Genetic: Pten*, *Dcx*, *Otx1* knock-out mice; Knock-in of human *Lis1*; *Congenital acquired*: *In utero* rat irradiation or alkylant agents (MAM, BCNU)
Tuberous sclerosis complex (TSC)	Cell specific-conditional *Tsc1* or *Tsc2* knock-out mice
Glioblastoma	Neocortical transplantation of human glioma cells in *scid* mouse or glioma cell lines in rat
INFECTIOUS	
Viral encephalitidies	Theiler murine encephalomyelitis virus
Cerebral malaria	Plasmodium berghei ANKA murine model
GENETIC OR PRESUMED GENETIC	
Spontaneous mutations	*Rat absence epilepsy*: GAERS, WAG/Rij, HVS; *Murine absence epilepsy*: Tottering, Lethargic, Stargazer, Mocha 2j, Slow-wave, Ent, Ducky, Gabrg2 conditional knock-in mutation
Induced monogenic mutations	Mouse knock-out or knock-in mutation of voltage-gated ion channels subunits (Na^+, K^+, Ca^{2+}), neurotransmitter receptor subunits (GABAA, nicotinic) and transporters, accessory synaptic proteins; cystatin B *(Cstb)* knock-out *www.informatics.jax.org/humanDisease.shtml*
Developmental epileptic encephalopathies	*Dravet syndrome*: *Scn1A*, *Scn1B*: Knock-in of human mutations in mice; Constitutive or conditional knock-out mice; *Infantile spams*: *Arx:* Knock-in of human mutations in mice; Constitutive or conditional knock-out mice; *APC:* Conditional knock-out mouse *SCN8A:* Knock-in of human mutations in mice

**Models apply to both rats and mice if not otherwise indicated. Mutiple hit rat model: doxorubicin+lipopolysaccharide±p-chlorophenylalanine.*

Abbreviations: APC, adenomatous polyposis coli; Arx, aristaless related homeobox; BCNU, carmustine 1-3-bis-chloroethyl-nitrosurea; Dcx, doublecortin; GAERS, Genetic absence epilepsy rat from Strasbourg;

Gabrg2, GABA-A receptor 2 subunit; HSV, high voltage spike-and-wave spindles; Lis1, lissencephaly 1; MAM, methylazoxymethanol; Otx,1 orthodenticle homeobox 1; Pten, phosphatase and tensin homolog; Scid, severe combined immundeficient; Scn1A,B, sodium voltage-gated channel (Nav1.1α,β subunit); SCN8A, sodium voltage-gated channel alpha subunit 8 (Nav1.6); TGF-β1, transforming growth factor-beta1; Tsc1-2, tuberous sclerosis complex 1,2; WAG/Rij, Wistar Albino Glaxo from Rijswijk.

Table 1.2.2. *Epileptogenesis models induced by an acquired brain injury (modified by Pitkanen and Lukasiuk, 2009).*

TYPE	MODEL	REFERENCES
Immature brain		
SE	Kainic acid (PN14)	(Stafstrom et al., 1992)
	Li-Pilocarpine (PN12 or PN21)	(Kubova et al., 2004; Marcon et al., 2009; Roch et al., 2002)
	Prolonged febrile seizures (PN10)	(Dube et al., 2006)
Mature brain		
SE	Kainic acid	(Ben-Ari and Lagowska, 1978; Mouri et al., 2008)
	Pilocarpine	(Turski et al., 1983)
	Li-pilocarpine	(Jope et al., 1986)
	Perforant pathway stimulation	(Mazarati et al., 1998)
	Intrahippocampal stimulation	(Lothman et al., 1989)
	Amygdala stimulation	(Mazzuferi et al., 2013; Nissinen et al., 2000)
Trauma	Lateral fluid percussion	(D'Ambrosio et al., 2004; Kharatishvili et al., 2006)
	CCI	(Bolkvadze et al., 2009; Clausen et al., 2009)
Stroke	Cortical photothrombosis	(Karhunen et al., 2007; Kelly et al., 2001)
	Cortical endothelin	(Karhunen et al., 2006)
	Cortical albumin	(Seiffert et al., 2004)

Animal studies have demonstrated the occurrence of molecular and cellular alterations, contributing to pathological outcomes such as spontaneous seizures, cognitive deficits, emotional impairment and drug-refractoriness.

Cellular and molecular alterations have been investigated and well characterized in the hippocampus since this region plays a pivotal role in neuronal hyperexcitability underlying seizures in various acquired models and in mesial TLE (Pitkanen and Lukasiuk, 2009). Moreover, the neuronal circuitry of the hippocampus is well known and easily accessible for

investigational studies. Figure 1.2.3 summarizes the most salient cellular and molecular alterations taking place during epileptogenesis in the hippocampus. It is important to consider that the epileptogenic process is likely not confined to hippocampus, but it may involve also other limbic and extralimbic areas (Jutila et al., 2002).

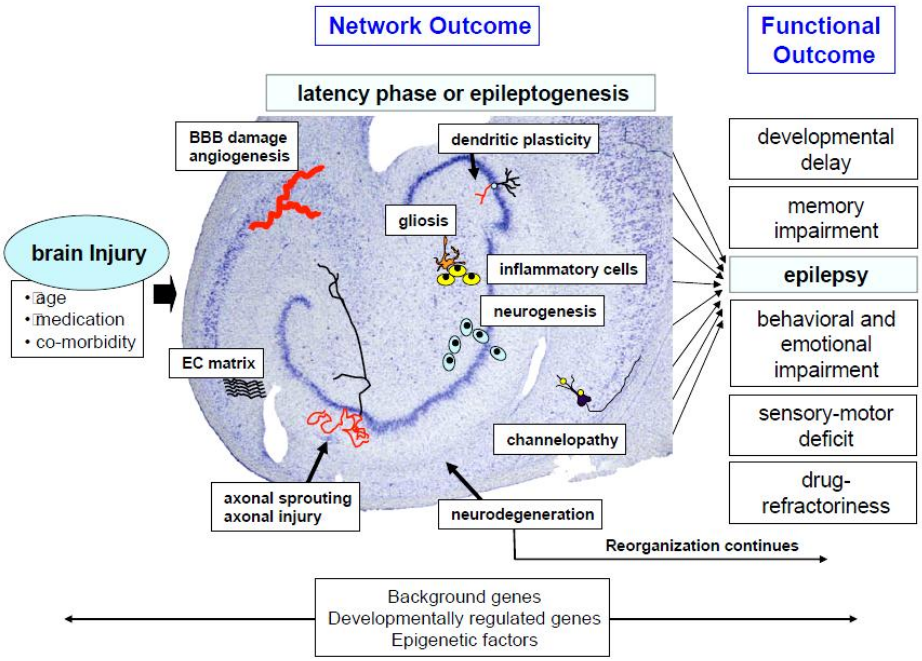


Figure 1.2.3. Cellular and molecular alterations occurring during epileptogenesis. *Brain injury (such as SE, TBI or stroke) triggers molecular, cellular and functional changes in the hippocampus, an area implicated in epileptogenesis in experimental models and in humans. The changes include neurodegeneration, neurogenesis, inflammation and gliosis, blood brain barrier (BBB) damage, reorganization of extracellular matrix, axonal sprouting, axonal injury, dendritic plasticity and alteration in voltage- and ligand-gated ion channels (channelopathy). These alterations may determine the generation of seizures and neurological deficits. The epileptogenic process is modulated also by epigenetic mechanisms and influenced by genetic background (Pitkanen and Lukasiuk, 2009).*

Neurodegeneration may occur after an epileptogenic insult. Damaged neuronal cells have been identified mostly in the hilus, where inhibitory interneurons play an important role in controlling the excitability of the hippocampus, and in cornu ammonis (CA) 1 pyramidal cell layer. Damage has been demonstrated also in CA3 pyramidal cell layer, and in parahippocampal cortices and amygdala. The reduction of neurodegeneration is apparently not associated with

an antiepileptogenic effect (Brandt et al., 2003), however it may alleviate behavioural deficits after SE (Brandt et al., 2006).

In animal models, increased **neurogenesis** has been detected within few days after SE (Parent, 2007) and it is has been reported also after stroke and TBI (Pitkanen and McIntosh, 2006). Aberrant neurogenesis consists of erroneous localization of newly-born neurons which can create abnormal and pathologic connections. There is evidence that altered neurogenesis is linked to learning and memory deficits as well as depression (Scharfman and Hen, 2007) and may contribute to epileptogenesis (Cho et al., 2015).

The search for pathogenic mechanisms of epileptogenesis has shown that **neuroinflammation** and reactive gliosis are common features in seizure-prone brain regions in various form of drug-resistant acquired epilepsies in humans and animal models (Aronica and Crino, 2011; Vezzani et al., 2011a). A more detailed description of neuroinflammation is given in Section 1.3.

Blood brain barrier (BBB) damage is a common feature of various epileptogenic insults such as SE, TBI and stroke (Friedman and Heinemann, 2012). Disruption of BBB determines brain extravasation of blood constituents, such as albumin, thus triggering transforming growth factor-β (TGF-β) mediated pathway in astrocytes. This activation induces modifications in astrocytes phenotype such as (1) reduced expression of potassium inward rectifying channels (Kir4.1) and water channels (aquaporin 4, AQP4), (2) reduced expression of gap junctions and (3) impaired glutamate metabolism, which compromises extracellular K^+ and water buffering and increases glutamate levels. These effects together with the induction of excitatory synaptogenesis may contribute to the generation of experimental seizures (Frigerio et al., 2012; Seiffert et al., 2004). Inhibition of this signaling reduces the incidence of epilepsy in animals exposed to albumin or injected with TGF-β or paraoxon (Bar-Klein et al., 2016; Weissberg et al., 2015), thus giving indication of a potential strategy for the prevention of post-injury epilepsy. Moreover, during BBB leakage leukocytes can migrate into the brain tissue. In particular, infiltrating blood-born monocytes were detected in the hippocampus after SE and

prevention of their entry into the brain dampened neuroinflammation, ameliorated neuronal damage and reduced BBB dysfunction (Fabene et al., 2008; Varvel et al., 2016).

Voltage- and ligand-gated ion channels are critical for regulating neuronal excitability (Lai and Jan, 2006). The properties of these channels are altered in epilepsy (Bernard et al., 2004; Noam et al., 2011), possibly leading to dysfunction of networks implicated in hyperexcitability and cognitive impairment. This phenomenon is called acquired **channelopathy** and affects dendritic, somatic or axonal channels. For example, hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) is decreased during epileptogenesis evoked by SE (Bernard et al., 2004; Noam et al., 2011). HCN1 are predominantly expressed in dendrites where they significantly modify dendritic intrinsic excitability as well synaptic potential shapes and integration. Indeed, reduced levels of HCN1 could lead to hyperexcitability and alterations in neuronal activity. On the contrary, SE induces transient and selective up-regulation of T-type Ca(2+) channels, causing up-regulation of cellular T-type Ca(2+) currents resulting in transient increase in intrinsic burst firing in CA1 pyramidal neurons, thus contributing to epileptogenesis (Becker et al., 2008).

Axonal and dendritic plasticity have been described after various epileptogenic events, including SE, TBI, stroke as well as in TLE models and patients (Isokawa, 2000; Mathern et al., 1996; Pitkanen et al., 2007). Sprouting of glutamatergic or gamma-aminobutyric acid (GABA)ergic axons in ectopic localization causes formation of abnormal connections between neurons, thus altering the neuronal network excitability. Loss of dendritic spines, reduced dendritic branches or changes in spine morphology can alter the availability of receptors for neurotransmitter, thus compromising the physiological flow of action potentials (Pitkanen et al., 2007).

The molecular changes observed during epileptogenesis after the inciting event show a pathway-specific temporal profile of induction, persistence and recovery. Pharmacological and genetic interventions targeting these molecules or pathways have shown disease-modifying effects thus demonstrating their implication in the pathogenic process. Among the molecules

implicated in epileptogenesis (Table 1.2.3) much attention has been devoted to those related to immunity and inflammation.

These molecular pathways may be linked to each other and the identification of nodal points to control them may help in the identification of targets for the development of drugs able to block the broad cascade of pathologic events taking place during epileptogenesis (Kobow et al., 2012).

Table 1.2.3. *Salient molecular pathways affected during epileptogenesis.*

MOLECULAR PATHWAYS	REVIEWS
BDNF/TrkB	(McNamara and Scharfman, 2012)
mTOR	(Ostendorf and Wong, 2015)
Adenosine/ADK	(Kochanek et al., 2006; Li et al., 2007)
TGF-β/ALK 5	(Cacheaux et al., 2009)
Neuroinflammation (e.g., IL-1β, HMGB1, TNF-α, COX2, prostaglandins, complement system)	(Vezzani et al., 2015)
Oxidative stress	(Rowley and Patel, 2013)
Extracellular matrix proteins	(Dityatev, 2010)

Abbreviations: BDNF, brain-derived neurotrophic factor; TrkB, tyrosine receptor kinase B; mTOR, mammalian target of rapamycin; ADK, adenylate kinase; IL-1β, interleukin-1β; HMGB1, high mobility group box 1; TNF-α, tumor necrosis factor-α.

The epileptogenic process is modulated also by the effect of genetic influence and epigenetic mechanisms.

Various genes are differentially expressed during epileptogenesis: unbiased microarray analysis of epileptogenic tissue in animal models identified common molecular pathways (Table 1.2.3) linked to known cellular changes (Figure 1.2.3) (Dingledine et al., 2017; Pitkanen et al., 2015). Individual genes show different temporal expression profiles after epileptogenic injuries, suggesting that targeted therapy should be adapted to the timing of occurrence and on the type of the epileptogenic insult.

Moreover, epigenetic modifications affect the expression of genes involved in neuronal homeostasis, excitability, cell survival and inflammatory processes (Lubin, 2012; Roopra et al., 2012). Epigenetic changes, such as modulation of DNA methylation, alteration in histone modifications and in microRNA (miRNA, small non coding RNA) expression, has been reported in experimental models and human epilepsy (Henshall and Kobow, 2015; Henshall et al., 2016; Roncon et al., 2015).

A global increase in hippocampal DNA methylation was correlated with spontaneous recurrent seizures in mice and a reversed DNA hypermethylation inhibited hippocampal sprouting of mossy fibers and prevented the progression of epilepsy for at least 3 months (Williams-Karnesky et al., 2013).

Sng and colleagues reported after kainic acid timely up-regulation in histone H3 phosphorylation in granule neurons and H4 acetylation throughout the hippocampus (Sng et al., 2006). Expression of histone deacetylases 2 is up-regulated in experimental and human epilepsy (Huang et al., 2002). The antiepileptic drug valproic acid by inhibiting histone deacetylases potently blocked seizure-induced neurogenesis and protected mice from seizure-induced cognitive impairment (Jessberger et al., 2007). Moreover, another histone deacetylase inhibitor administered in combination with diazepam reduced neurodegeneration after SE (Rossetti et al., 2012). However, the role of histone deacetylase inhibitors in seizure generation is still controversial (Henshall and Kobow, 2015).

Several studies identified changes in more than 100 different miRNAs in rodents and in human TLE hippocampi during epileptogenesis, including miRNAs targeting dendritic spines (e.g., miR-134), neuronal survival (e.g., miR-34a, miR132 and miR184), neurotransmitter receptors, transcriptional regulators and modulators of the inflammatory response (e.g., miR-146a) (Henshall et al., 2016; Iori et al., 2017a). Interventions with either small molecule inhibitors (antagomiR) or oligonucleotides that mimic miRNAs allowed to determine their functional role in epileptogenesis. Antagonizing miR-134 (Jimenez-Mateos et al., 2012; Reschke and Henshall, 2015) or mimicking miR-146a (Iori et al., 2017b) resulted in strong inhibition of epileptogenesis

in animal models. miRNAs target also neuron-restrictive silencer factor (NRSF/REST) and are in turn modulated by NRSF. This factor controls the transcription of genes for voltage-gated Na^+ and K^+ channels, such as HCN1 (McClelland et al., 2011). NRSF is upregulated during epileptogenesis which may drive down-regulation of HCN1. Moreover, NRSF blockade restored electrophysiological properties in neurons (McClelland et al., 2011).

Although *in vivo* targets of miRNAs linked to epilepsy remain to be clearly identified, these data indicate that miRNAs are nodal point of control of broad epileptogenic pathways.

1.3 NEUROINFLAMMATION – general aspects

Inflammation is a defensive response to harmful stimuli, resulting from traumatic or infectious tissue injury. Cells of innate immunity (neutrophils, monocytes and NK cells) drive the first line of defense against dangerous stimuli and generate the acute inflammatory response. The part of the immune system (T and B cells) that specifically recognizes antigens from pathogens and prepares the body for current and future challenges is named **adaptive immunity**. Failure or ineffective resolution of the inflammatory phenomenon may result in an amplified or persistent inflammatory response leading to tissue dysfunction. Dysregulated inflammatory response has been identified in various pathologies including diabetes, psoriasis, rheumatoid arthritis, autoimmune diseases, cardiovascular, cerebrovascular and metabolic disorders, as well as some forms of cancer and acute and chronic neurodegenerative pathologies (Serhan et al., 2008).

In the brain, the innate and adaptive immune responses are triggered not only during infections or in autoimmune diseases, but also after acute injuries such as brain trauma, ischemia (Allan and Rothwell, 2001; Lucas et al., 2006), in epilepsy and in chronic neurodegenerative disorders (Aarli, 2000; Bauer et al., 2001; Nguyen et al., 2002). In epilepsy, a significant component of this innate inflammatory response is confined to brain resident cells (e.g., microglia, astrocytes, neurons and endothelial cells of the BBB) and is defined

neuroinflammation (Vezzani et al., 2011a). In particular, a prominent role in the **innate immune response** is played by microglia and astrocytes (Farina et al., 2007; Lehnardt, 2010). These cells produce and release a large number of pro-inflammatory molecules, such as cytokines, chemokines and adhesion molecules, to "sense" the presence of offending agents that alter brain homeostasis, thereby promoting tissue healing mechanisms. The inflammatory response is triggered during infections by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) the major component of the outer membrane of Gram-negative bacteria or by endogenous molecules released by damaged and stressed cells called DAMPs (damage-associated molecular patterns), such as adenosine triphosphate (ATP), S100 calcium binding protein β (S100β) and HMGB1. The innate immune receptors for PAMPs and DAMPs are the Toll-like receptors (TLRs), NOD-like receptors, RIG-I-like receptors, AIM2-like receptors, and the receptor for advanced glycation end products (RAGE). Besides their role in immunity-related functions (Hanisch and Kettenmann, 2007), glial cells are involved in modulating neuronal functions such as guiding cell migration during brain development, modulating synaptic function and plasticity, regulating the extracellular microenvironment by controlling ion, neurotransmitter and water concentration and modulating BBB permeability (Friedman et al., 2009; de Lanerolle et al., 2010).

1.3.1 Astrocytic function and activation

Two principal astrocytic populations have been identified in the rodent hippocampus. Astrocytes expressing glutamate transporter are enriched in glial fibrillary acidic protein (GFAP, a cytoskeletal protein), have irregular cell bodies and branched processes, and are involved prominently in glutamate re-uptake and gap-junction coupling. Astrocytes expressing glutamate receptors (GluRs) are characterized by low GFAP expression, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), low glutamate uptake and no gap-junction coupling (Matthias et al., 2003).

Astrocytes are interconnected via gap junctions to form a large intercellular network. This connection allows the dissipation of ions and metabolites that could be detrimental if they accumulate in the extracellular space (Pekny and Nilsson, 2005). Furthermore, due to expression on astrocyte membranes of ion channels for K^+ such as Kir and the AQP4, these cells regulate the extracellular microenvironment, modulating ion concentration and controlling the volume and the osmolarity of the extracellular space (Devinsky et al., 2013; Farina et al., 2007). In physiological conditions to maintain osmotic balance, K^+ ions are carried inside the cells accompanied by water entry through AQP4.

The presence of a functional astrocyte network mediated by intracellular Ca^{2+} oscillations has also been reported (Bezzi et al., 2001; Jabs et al., 2008; Volterra and Meldolesi, 2005). In response to increased intracellular Ca^{2+} due to neuronal stimulation, glial cells are able to modulate synaptic transmission releasing gliotransmitters, such as cytokines, D-serine, ATP, glutamate and GABA acting on neuronal receptors (Devinsky et al., 2013; Farina et al., 2007). Thus, the communication between neurons and astrocytes is bidirectional and astrocytes are part of the "tripartite synapse" together with pre- and post-synaptic terminals of neurons (Wetherington et al., 2008). Moreover, glutamate uptake by astrocytes is essential for preventing spill-out from the synaptic cleft and activation of peri-synaptic/extra-synaptic excitatory glutamate receptors which may determine excitotoxicity and increase excitability (Wetherington et al., 2008).

Astrocytes contribute also to BBB integrity because their endfeet wrap around endothelial cells which are connected via tight junctions to constitute the barrier. Furthermore, they regulate the movement of water and molecules between the blood and the brain parenchyma (Ballabh et al., 2004; Pachter et al., 2003). At the same time, astrocytes can alter BBB permeability releasing chemical signals such as pro-inflammatory chemokines and cytokines which interact with their cognate receptors expressed by endothelial cells, thus altering their physiological state (Morin-Brureau et al., 2011).

Following injury, astrocytes become activated and proliferate with hypertrophy of cellular processes (defined astrogliosis) and up-regulation of GFAP protein (John et al., 2005; Moynagh, 2005). Astrogliosis can create a physical barrier, termed the glial scar, which isolates the damaged tissue from healthy cells (Raivich et al., 1999). Besides this beneficial effect, the glial scar can also block axonal regeneration, restricting the neuronal outgrowth at the brain injury site (Bush et al., 1999; John et al., 2005; Pekny and Nilsson, 2005). Astrocytes may contribute to sustain inflammation, which in turn reduces their homeostatic functions (Devinsky et al., 2013).

1.3.2 Microglia function and activation

Microglia are brain-resident macrophage-like cells that play a central role as phagocytes and mediators of innate immune response (Kreutzberg, 1996).

In physiological conditions, microglia assume a ramified and resting phenotype monitoring the microenvironment homeostasis, continuously moving their processes. Maintaining microglia in a relatively quiescent state is, in part, due to signals derived from neuronal- and astrocytic-released factors (Figure 1.3.1) (Hanisch and Kettenmann, 2007).

Microglia can rapidly respond to various microenvironmental changes because they express different types of receptors on their membranes: (a) purinergic P2X7 receptors respond to ATP, which is released by cells following brain injuries (Garden and Möller, 2006); (b) neurotransmitter receptors for glutamate, GABA and monoamines (Hanisch and Kettenmann, 2007); (c) receptors for cytokines (IL-1β), danger signals, chemokines, bacterial or viral constituents, *etc* that play a critical role in initiating and regulating the immune response (Garden and Möller, 2006).

Among the different chemokines used in the communication between neurons and microglial cells, chemokine CX3CL1 (fractalkine) and its receptor CX3CR1 deserve special attention. In the CNS, fractalkine is expressed by neurons, while its receptor is expressed uniquely by microglia

(Wolf et al., 2017). Fractalkine-mediated neuron-microglia signaling modulates numerous physiological processes, including the maturation of synaptic connections, neuronal survival, synaptic transmission and plasticity. Mice lacking fractalkine signaling display deficits in hippocampal-dependent learning and memory (Rogers et al., 2011; Zhan et al., 2014) and in microglial development in the hippocampus (Pagani et al., 2015).

After detecting pathological signals, microglia respond by transforming their morphology to a round-shape with thickened and short processes (Figure 1.3.1) (Kreutzberg, 1996). Activated microglia can assume different phenotypes resulting in diverse functional properties depending on the nature of the stimulus, its intensity and duration. For example, following LPS, β-amyloid aggregates or a high amount of interferon-γ (IFN-γ), microglia acquire a cytotoxic and phagocytic capacity (Hanisch and Kettenmann, 2007). Activated pro-inflammatory microglia (David and Kroner, 2011), produce molecules, such as cytokines and prostaglandins. On the other hand, after IL-4 or low doses of IFN-γ, microglia can support neurogenesis and offer neuroprotection secreting anti-inflammatory factors or removing extracellular glutamate (Hanisch and Kettenmann, 2007). These microglial cells express high levels of IL-10 and TGFβ, show defective nuclear factor-kappa B (NF-κB) activation and downregulate expression of pro-inflammatory cytokines (David and Kroner, 2011).

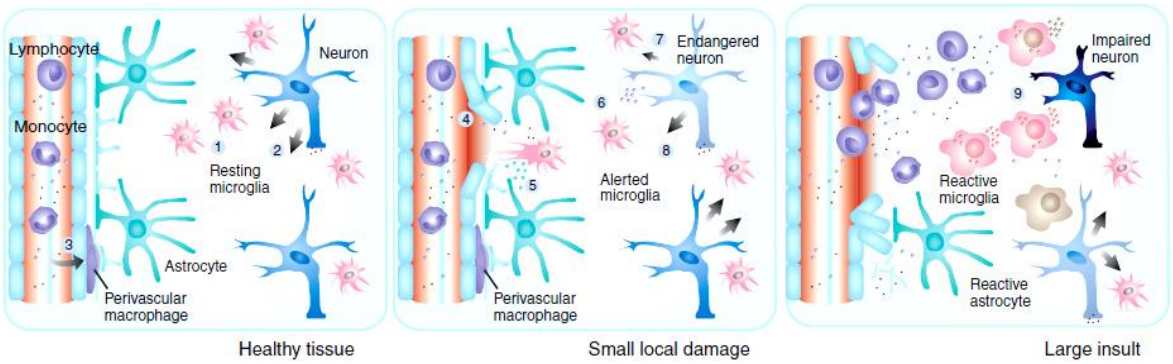


Figure 1.3.1. The activation state of microglia. *Left, resting microglia in healthy tissue constantly screening their environment. They can immediately sense alteration of structural and functional integrity because they express receptors for different molecules (1). Neurons deliver signals which keep microglia in their resting mode (2). Besides the parenchymal microglia, there are also perivascular macrophages in closer association with blood vessels (3). Middle, upon detection of minute homeostatic alterations, microglia can rapidly respond with a reorganization of processes and a change in the activity profile (4). The response is probably supported by neighboring astrocytes releasing, for example, ATP (5). Microglia can produce neurotrophic factors to support endangered neurons (6). Damaged neurons blocking the release of resting signals or emitting signals indicating disturbed functions call for microglial assistance (7, 8). Microglial cells may be able to limit further damage and restore normal homeostasis. Right, stronger insults to the CNS (infection or tissue injury) may trigger more drastic changes in the functional microglia phenotype. Depending on the nature of the stimuli and their context, microglial cells need to acquire and adapt their behaviour. Excessive acute, sustained or maladaptive responses of microglia may lead to substantial impairment of neurons and glia (9) (Hanisch and Kettenmann, 2007).*

Microglial and astrocytic responses are versatile and vary depending on the microenvironment, the nature, intensity and duration of the stimulus (Hanisch and Kettenmann, 2007; Pekny and Nilsson, 2005). The transient and focal activation of microglia and astrocytes could be beneficial, limiting and repairing the damaged tissue. However, excessive and maladaptive activation of glial cells may lead to a sustained neuroinflammation that may progress towards a pathological phenomenon (Hanisch and Kettenmann, 2007; Schwartz et al., 2006).

1.4 NEUROINFLAMMATION IN EPILEPSY

1.4.1 Neuroinflammation in experimental models

Epileptogenic events and seizure recurrence can induce and perpetuate a persistent inflammatory state in the brain. Experimental models have shown that this inflammatory process is involved in the mechanisms underlying the precipitation and recurrence of seizures, and in the associated cell loss and comorbidites (Vezzani et al., 2011a, 2017).

Microglia and astrocyte activation and the resulting neuroinflammation in brain regions affected by a pathologic event has been shown in models of acute seizures and pharmacoresistant epilepsy (Terrone et al., 2016). Recent evidence supports the role of neuroinflammation in epileptogenesis following SE, TBI, stroke, febrile seizures, as well as in genetic models of absence seizures, such as in GAERS rats (Akin et al., 2011), in models of progressive myoclonus epilepsy (Okuneva et al., 2015) and in a mouse model of tuberous sclerosis (TSC) (Wong and Crino, 2012). In animal models, the number of spontaneous seizures correlates with the extent of microglia and astrocyte activation (Filibian et al., 2012; Ravizza et al., 2008a). In particular, astrocytic activation assessed by measuring myo-inositol by MRI spectroscopy and by S100β immunohistochemistry, correlates with the extent of cell loss and the frequency of spontaneous seizures in epileptic rats (Filibian et al., 2012). A recent paper by Pascente demonstrates that the persistent increase of myo-inositol levels in the hippocampus predicts which rats develop epilepsy in a model of SE induced by pilocarpine (Pascente et al., 2016).

Also neurons and endothelial cells of the BBB produce pro-inflammatory mediators during epileptogenesis and in chronic epilepsy (Bien et al., 2012; Iyer et al., 2010; Vezzani et al., 2011a). In addition, leukocytes may contribute to the inflammatory state. In particular, these infiltrating cells have been detected in the hippocampus after SE induced by administration of pilocarpine (Fabene et al., 2008) or intrahippocampal injection of kainic acid in mice (Zattoni et al., 2011) although their role in epileptogenesis is still unclear.

In epilepsy models, the broad inflammatory response is activated by the release of DAMPs (e.g., HMGB1, S100β and ATP) and cytokines (e.g., IL-1β and TNF-α) by injured or activated brain cells. DAMPs and related inflammatory mediators activate their receptors expressed by glia, neurons and endothelial cells of the BBB, thus resulting in activation of intracellular signalling pathways and mediating different effects depending on the cell type (Vezzani et al., 2011a, 2013). Activation of **microglia and astrocytes** determines NF-kB-dependent transcriptional up-regulation of inflammatory genes, thus resulting in the perpetuation of the inflammatory state and affecting also neuronal functions (Devinsky et al., 2013).

Activation of receptors on **endothelial cells** induces up-regulation of adhesion molecules for the recruitment of circulating leukocytes and contributes to breakdown of tight junction and BBB leakage; this phenomenon leads to extravasation of albumin, thus activating TGF-β signaling in astrocytes and inducing their dysfunctional phenotype (see Section 1.2.3) (Friedman et al., 2009; Frigerio et al., 2012).

Activation of **neuronal receptors**, which are induced in diseased tissue although not expressed at measurable levels in physiological conditions, modifies Ca^{2+} influx, thus resulting in neuronal hyperexcitability, and promotes cell injury and network reorganization (Balosso et al., 2014; Viviani et al., 2003). Inflammatory mediators also modify voltage- or receptor-gated ion channel function (Viviani et al., 2007) and promote changes in neuronal glutamate [N-methyl-D-aspartic acid (NMDA) and AMPA receptor] and GABA receptor expression, subunit composition and function (Balosso et al., 2009; Frasca et al., 2011; Roseti et al., 2013, 2015), thus determining an imbalance between the excitatory and the inhibitory transmission. These direct effects on neurons may play a crucial role in seizure generation and recurrence as shown and outlined in Figure 1.4.1.

The next paragraphs describe pivotal pathways activated during epileptogenesis, namely IL-1β/IL-1R1, HMGB1/TLR4, COX2/PGE and TNF-α/P55/P75, and the pharmacological interventions aimed at blocking these pathways during epileptogenesis or in models of acute and chronic seizures.

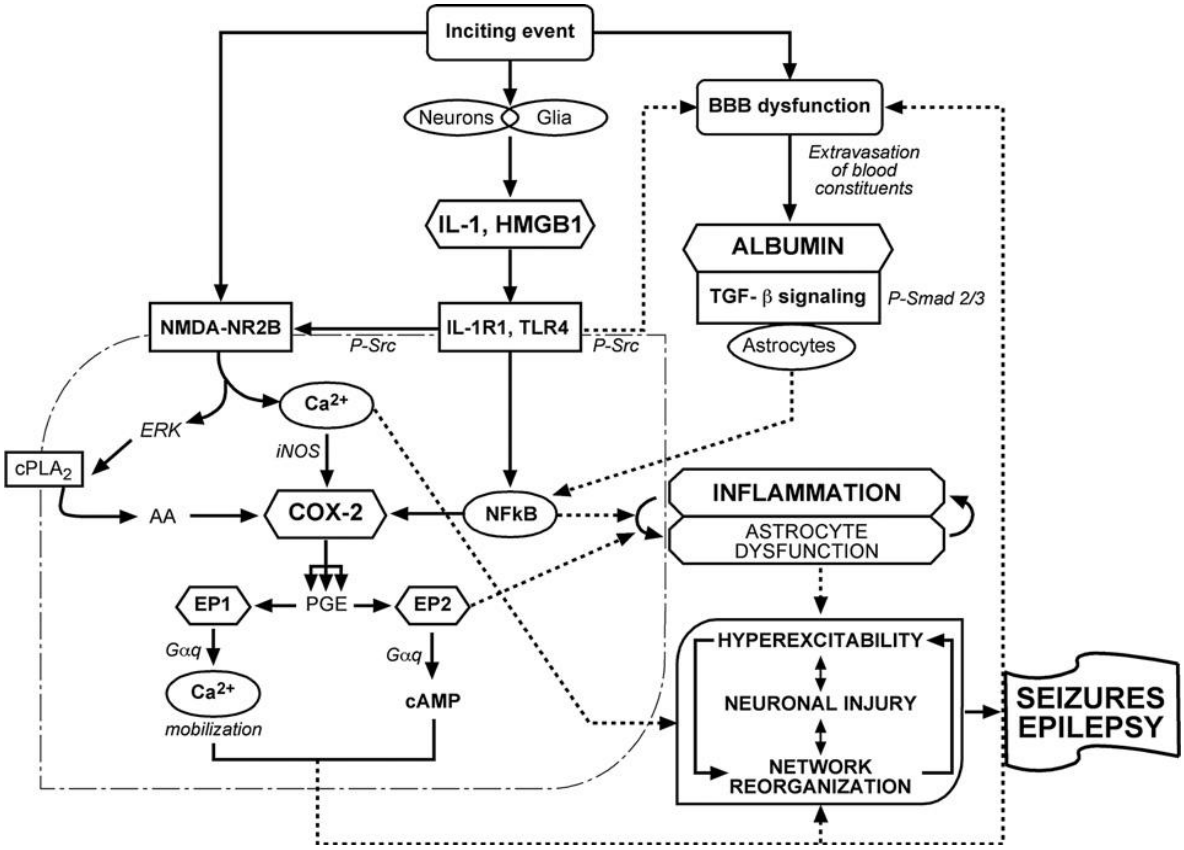


Figure 1.4.1. Schematic drawing of the pathophysiological cascade mediated by three key pro-inflammatory pathways activated in epilepsy: IL-1R1/TLR4, COX-2 and TGF-β. *Epileptogenic insults may lead to activation of microglia, astrocytes and neurons, and/or to BBB dysfunction. Primed cells release cytokines such as IL-1β, and danger signals such as HMGB1, thereby eliciting pro-inflammatory effects in the target cells via activation of IL-1R1/TLR4. Signaling activation in neurons results in a rapid increase of NMDA receptor Ca^{2+} conductance via Src-mediated phosphorylation of the NR2B subunit, leading to increased intracellular Ca^{2+}, which in turn results in neuronal hyperexcitability or injury, decreased seizure threshold, network reorganization. Src activation in endothelial cells may contribute to BBB breakdown. IL-1R1/TLR4-mediated activation of the NF-kB-depedent transcription of genes contributes both to molecular and cellular changes involved in epileptogenesis, and to perpetuate inflammation. Inciting events may also lead to glutamate-mediated activation of NMDA receptors in neurons which in turn promotes COX-2 activation via inducible nitric oxyde synthase (iNOS) and Phospholipase A2 (PLA2)-mediated production of arachidonic acid (AA). Prostaglandin E (PGE) is produced and activates EP1 and EP2 receptors coupled to intracellular signaling involving Ca^{2+} mobilization from intracellular stores and cAMP production, respectively, also contributing to inflammation, BBB dysfunction and pathologic outcomes. BBB dysfunction resulting from the inciting event or from inflammation in turn leads to albumin extravasation in the brain, and subsequent activation of TGF-β in astrocytes. This signaling promotes further inflammation by NF-kB dependent gene transcription and astrocitic dysfunction. This chain of consequential or concomitant events gives raise to neuronal network hyperexcitability, cell injury, and network reorganization which are then responsible for the onset of seizures and the development of epilepsy (Vezzani et al., 2013).*

1.4.1.1 IL-1β, IL-1R1 and IL-1Ra

The cytokine **IL-1β** is a pivotal mediator of neuroinflammation following epileptogenic injuries and during seizures (Vezzani et al., 2011b). mRNA and protein levels of IL-1β are rapidly increased (≤30 min) after the induction of electrically-induced SE (De Simoni et al., 2000). This up-regulation persists during epileptogenesis and in the chronic phase of epilepsy (De Simoni et al., 2000). Immunohistochemical studies confirmed IL-1β up-regulation (Ravizza et al., 2008b) and demonstrated that during the acute phase of SE, IL-1β is produced by both microglia and astrocytes whereas astrocytes are mainly involved during epileptogenesis. These results have been confirmed in other SE models of epileptogenesis (Bartfai et al., 2007; Dube et al., 2010; Marcon et al., 2009; Noe et al., 2013).

In physiological condition, the cytokine is stored in the cytoplasm in its biologically inactive form (pro-IL-1β). Following inflammasome activation, pro-IL-1β is cleaved by IL-1β converting enzyme (named ICE/Caspase-1) that produces the mature and active IL-1β that can be released extracellularly (Dinarello, 2005; Fantuzzi and Dinarello, 1999). ICE/Caspase-1 is part of the inflammasome, a multiprotein complex required also for the release of HMGB1. Extracellular ATP mediates the stimulation of P2X7 receptors which is pivotal for ICE/Caspase-1 activation (Vezzani et al., 2011b, 2013). Deletion of the ICE/Caspase-1 gene results in a delayed seizure onset and a reduction in seizure frequency after kainic acid injection in mice (Ravizza et al., 2006a).

IL-1R1 mediates the biological effects of IL-1β. The receptor is barely detectable in control brain, but it is rapidly upregulated in hippocampal neurons and astrocytes during epileptogenesis. This pattern of neuronal and astrocytic IL-1R1 expression persists in chronic epileptic tissue (Ravizza et al., 2006a, 2008b). Thus, IL-1β produced and released by glial cells after the initial trigger activates IL-1R1 in neurons and astrocytes, indicating that the cytokine has both autocrine and paracrine effects thus establishing functional communication between glia and neurons (Vezzani et al., 1999a, 2002). Increased IL-1β and IL-1R1 immunoreactivity was

found during epileptogenesis in perivascular astrocytic endfeet impinging on blood vessels and in endothelial cells of the microvasculature. This increased expression is associated with enhanced BBB permeability to serum albumin (Ravizza et al., 2008a; Seiffert et al., 2004; van Vliet et al., 2007) that can contribute to neuronal hyperexcitability (Oby and Janigro, 2006; Seiffert et al., 2004). Transgenic mice lacking the IL-1R1 gene were intrinsically less susceptible to seizures (Vezzani et al., 2000).

These modifications in animal models have been validated in tissue from TLE patients where IL-1β and IL-1R1 were broadly expressed by astrocytes, microglia and neurons (Ravizza et al., 2008a). Moreover, in TLE brain specimens there is evidence of: (1) activation of IL-1β/IL-1R1 mediated pathway by up-regulation of interleukin 1 receptor associated kinase 1 (IRAK) (Roseti et al., 2015) and (2) induction of NLRP1, NLRP3 inflammasome and ICE/Caspase-1 (Henshall et al., 2000; Meng et al., 2014; Tan et al., 2015).

The production of cytokines is accompanied by the concomitant synthesis of anti-inflammatory mediators apt to modulate the inflammatory response and to prevent the occurrence of deleterious effects. In particular, **IL-1Ra**, an endogenous competitive antagonist of IL-1R1, is increased after SE to a lesser extent than IL-1β and with a delayed time-course (De Simoni et al., 2000). This pattern of induction differs from the peripheral immune response to infection, where IL-1Ra is produced 100-1000 fold in excess as compared to IL-1β (Dinarello, 1996), suggesting that the brain is less effective to induce a crucial mechanism able to inhibit the deleterious effects of IL-1β. The human recombinant form of IL-1Ra, anakinra, is used for the treatment of rheumatoid arthritis in adult patients and was recently used in humans to control pharmacoresistant seizures (see Section 1.4.2) (Jyonouchi, 2016; Kenney-Jung et al., 2016).

1.4.1.2 HMGB1 isoforms and TLR4

HMGB1 is a mediator of sterile neuroinflammation evoked by epileptogenic injuries (Maroso et al., 2010; Vezzani et al., 2011b). HMGB1 is a highly conserved non-histone nuclear protein expressed by most eukaryotic cells where it binds to chromatin (Yang et al., 2005) and regulates gene transcription (Scaffidi et al., 2002). HMGB1 has several isoforms, each of which has distinct physiological and pathological functions. Non-acetylated HMGB1 is released passively from necrotic cells (Scaffidi et al., 2002). Acetylation of key lysine residues in HMGB1 indicates active release during inflammation, a step which requires nucleus-to-cytoplasm translocation (Lamkanfi et al., 2010; Yang et al., 2005). Furthermore, the redox state of the protein determines its receptor interactions: under basal conditions, HMGB1 is predominantly fully-reduced inside the cell, but can be oxidized by reactive oxygen species upon translocation to the cytoplasm or after its extracellular release. Redox modification of three key cysteine residues, C23, C45 and C106, determines the functional activity of HMGB1 (Figure 1.4.2 and Table 1.4.1). In particular, disulfide HMGB1, containing an intramolecular disulfide bond between C23 and C45 and a reduced C106 (Yang et al., 2012), specifically binds and signals *via* the TLR4 to induce cytokine release in macrophages (Schiraldi et al., 2012), microglia and astrocytes (Maroso et al., 2010; Weber et al., 2015; Zurolo et al., 2011). Reduced HMGB1, in which all three cysteines are reduced, forms a heterocomplex with the C-X-C motif chemokine 12 (CXCL12) and binds CXCR4 to initiate chemotaxis (Venereau et al., 2012). HMGB1 that is terminally oxidized to sulfonyl groups on all cysteines (sulfonyl HMGB1) does not affect cell migration or cytokine induction (Figure 1.4.2 and Table 1.4.1) (Yang et al., 2013). The disulfide and reduced isoforms have mutually exclusive functions. HMGB1 also activates RAGE but the binding efficiency of each isoform for RAGE is still unresolved.

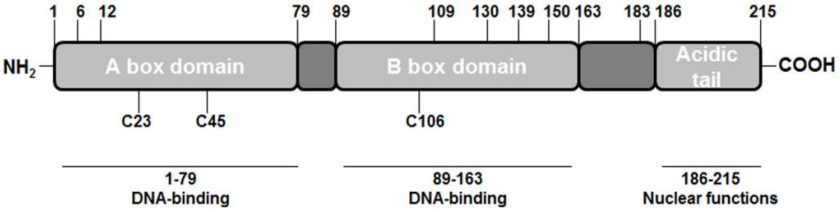
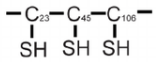
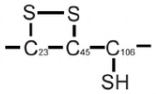
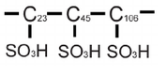


Figure 1.4.2. HMGB1 structure *(Ranzato et al., 2012).*

Table 1.4.1. *Redox isoforms of HMGB1 and their biological activity.*

HMGB1 species	Schematic overview	Cytokine/chemoattractant reaction
Reduced-HMGB1		Chemoattractant activity
Disulfide-HMGB1		Cytokine activity
Fully oxidized HMGB1		No cytokine or chemoattractant activity

TLR4 shows constitutive low expression in control condition *in vivo*; however, this receptor is rapidly up-regulated after seizure onset and in the chronic epileptic phase in both neurons and astrocytes, but TLR4 was undetectable in microglial cells (Maroso et al., 2010). Furthermore, total HMGB1 and its nucleus-to-cytoplasm translocation increases in neurons and glia in human drug-resistant epileptic *foci* (Maroso et al., 2010; Zurolo et al., 2011), and the corresponding animal models (Maroso et al., 2010). In addition, mice with a functional inactivation of TLR4, due to a spontaneous mutation in the cytosolic domain, had a delay in kainic acid–induced seizure onset and a substantial reduction in seizure frequency and total duration (Maroso et al., 2010). Disulfide HMGB1 is the isoform that promotes seizures and cell loss (Balosso et al., 2014; Iori et al., 2013; Maroso et al., 2010).

1.4.1.3 IL-1β/IL-1R1 and HMGB1/TLR4 axes

IL-1R1 and TLR4 share a cytosolic domain named the Toll/IL-1 receptor (TIR) domain (O'Neill and Bowie, 2007). Binding of agonists to the receptors and subsequent recruitment of MYD88 and other adaptor proteins, activates cell signaling via IRAK1/4 and TRAF6 or mitogen associated protein kinases (MAPKs) to induce expression of genes involved in inflammation, under the respective control of NF-kB and Activator protein (AP)-1 (O'Neill and Bowie, 2007). In addition to the slower transcriptional effects in astrocytes, microglia and neurons (Davis et al., 2006), the activation of IL-1R1/TLR4 results in a rapid post-translational effect in neurons. The binding of IL-1β/HMGB1 to IL-1R1/TLR4 induces sphingomielinase-mediated ceramide production. This cascade of events activates Src Kinase-mediated phoshorylation of the NR2B subunit of NMDA receptors, thus increasing Ca^{2+} influx (Figure 1.4.3) (Balosso et al., 2008, 2014; Viviani et al., 2003). This post-translational molecular event underlies the pro-ictogenic activity of both IL-1β and HMGB1, as well as their excitotoxic properties (Allan et al., 2005; Balosso et al., 2008, 2013; Iori et al., 2013; Viviani et al., 2003). A recent paper described that the activation of TLR4 by HMGB1 increased afferent evoked dentate gyrus excitability after concussive brain injury in mice (Li et al., 2015). Finally, both IL-1β and HMGB1 have been reported to increase the extracellular glutamate levels by inhibiting glutamate re-uptake (GLT-1 down-regulation) and promoting its release from glia or by enhancing NMDA-mediated glutamate release from synaptic terminals, thereby increasing neuronal excitability (Iori et al., 2016; Pedrazzi et al., 2006, 2012).

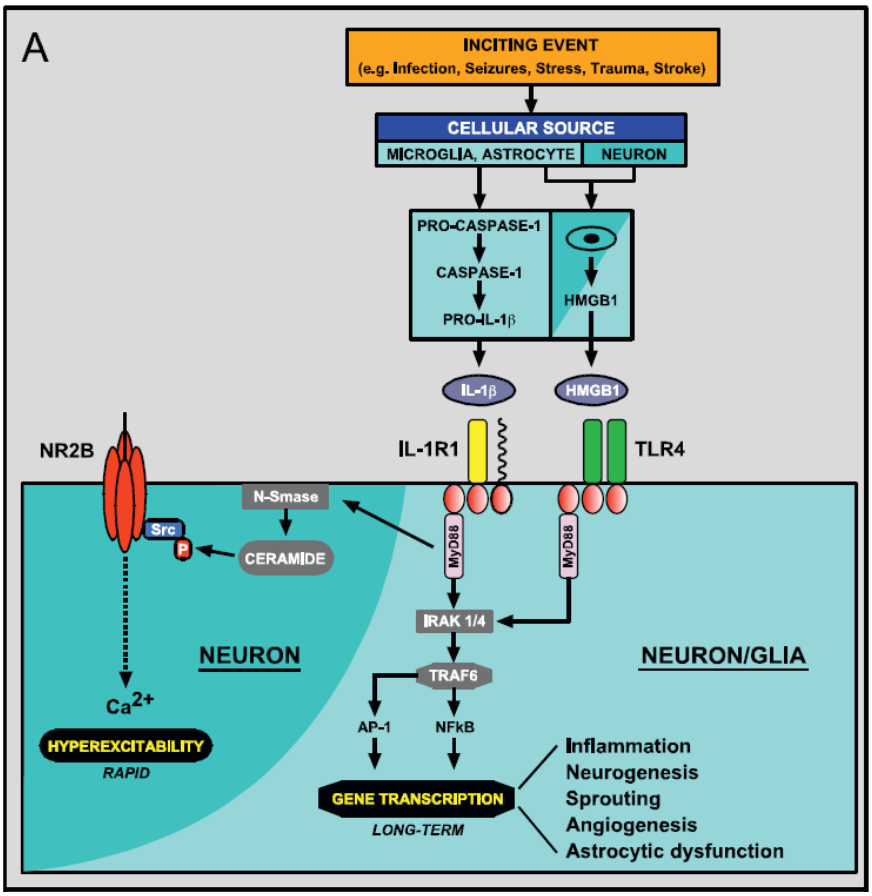


Figure 1.4.3. Schematic drawing of the pathophysiological cascade mediated by IL-1R1 and TLR4 during epileptogenesis. *An inciting event determines the production and release of cytokines, such as IL-1β, and danger signal, such as HMGB1, from microglia, astrocytes and neurons. Binding of IL-1β and HMGB1 to their respective receptors IL-1R1 and TLR4 determines through the activation of intracellular signaling cascades: (a) the phosphorylation of NR2B subunit of NMDARs, thus increasing hyperexcitability in neurons; (b) activation of transcription of genes associated with inflammation, neurogenesis, sprouting, angiogenesis and astrocytic disfunction in glia and neurons (Vezzani et al., 2011b).*

1.4.1.4 COX-2

Among the chemical mediators determining the establishment and propagation of a neuroinflammatory state, **cyclooxygenase-2** (COX-2) and the **prostaglandins** (PGs) play a pivotal role. PGs are lipid autacoids derived from arachidonic acid (AA); they sustain homeostatic functions but at the same time they can mediate pathogenic mechanisms (Ricciotti and FitzGerald, 2011).

Cyclooxygenases-1 and -2 are membrane-associated proteins that catalyze the conversion of AA to prostaglandin H2 (PGH_2). PGH_2 in turn is rapidly converted to one of five prostanoids: thromboxane A2, PGF_{2a}, PGE_2, prostacyclin (PGI_2), or PGD_2.

In experimental epilepsy models, the expression of COX-2 enzyme is induced rapidly in neurons and astrocytes (Rojas et al., 2014). Conditional ablation of COX-2 in forebrain neurons is neuroprotective and reduces neuroinflammation after SE (Serrano et al., 2011).

As previously mentioned, the substrate of COX-2 for the production of PGs is AA. This lipid is principally produced in the brain by monoacylglycerol lipase (MAGL), a key enzyme in the hydrolysis of the endocannabinoid 2-arachidonoylglycerol (2-AG) to AA.

1.4.1.5 TNF-α

TNF-α plays a role in modulating neuronal excitability and seizure threshold. Evidence of increased production of TNF-α in the brain has been reported for kainic acid-induced seizure (Lehtimaki et al., 2003) and following electrically-induced seizures and SE (De Simoni et al., 2000; Plata-Salaman et al., 2000; Shandra et al., 2002). TNF-α can have both pro- and anti-convulsant effects, depending on the receptor activated (p55 or p75) and the intracellular pathway related (Balosso et al., 2005, 2009; Weinberg et al., 2013). In 2005, Balosso showed that increased brain levels of TNF-α resulted in significant inhibition of seizures in mice, and this action was mediated by neuronal p75 receptors (Balosso et al., 2005). **TNF-α receptors** are barely detectable in normal brain tissue but they are rapidly up-regulated in microglia and neurons after electrically-induced seizures and after kainic acid injection in mouse hippocampus (De Simoni et al., 2000; Vezzani et al., 2002). In 1996, Bruce has demonstrated that mice lacking TNF-α receptors showed a reduced microglial activation following kainic acid injection indicating that TNF-α signaling plays an important role in microglial response to injury (Bruce et al., 1996). These data indicate that TNF-α, as well as IL-1β, can act as a soluble

mediator of functional glio-neuronal communication (Beattie et al., 2002; Bezzi et al., 2001; Stellwagen et al., 2005).

In general, the effect of cytokines on glial and neuronal function depends on their concentration, the tissue exposure time, the type of cells producing and responding to it, and the microenvironment in which the neuroinflammatory phenomenon is established (Bernardino et al., 2005).

1.4.2 Clinical evidence of brain inflammation in human epilepsy

Clinical evidence demonstrates the presence of pro-inflammatory signaling activation in brain specimens, blood and cerebrospinal fluid (csf) from pediatric and adult patients affected by epilepsy of various etiologies (Aronica and Crino, 2011).

Immunohistochemical analysis of brain tissue from patients with different types of refractory epilepsy (TLE, Rasmussen's encephalitis, TSC and cortical developmental malformations) revealed the presence of an inflammatory state in the epileptogenic areas similar to animal models (Boer et al., 2006; Maroso et al., 2010; Ravizza and Vezzani, 2006; Ravizza et al., 2006b, 2008a; Vezzani et al., 2011a). Interestingly, the extent of inflammation sustained by glial cells, or the number of microglial cells activated in the epileptogenic areas, correlates positively with the frequency of seizures and the duration of epilepsy before surgery in patients (Aronica et al., 2005; Boer et al., 2008; Ravizza and Vezzani, 2006).

In human TLE hippocampi a pro-inflammatory phenotype has been detected in glia and neurons: NF-kB overexpression, activation of IL-1β and IL-1R1, overexpression of TLR4 and translocation of HMGB1, and induction of the complement system. These changes are associated with BBB leakage and albumin extravasation in brain tissue (Aronica et al., 2007; Maroso et al., 2010; Ravizza et al., 2008a; Rigau et al., 2007; van Vliet et al., 2007). IL-1β and IL-1R1 are expressed also by perivascular astrocytic endfeet impinging on blood vessels and in endothelial cells of microvasculature, thus suggesting an involvement of this pathway in BBB

dysfunction similar to animal models (Ravizza et al., 2008a; Seiffert et al., 2004; van Vliet et al., 2007). In contrast to the prominent glia activation and the presence of macrophages, relatively few lymphocytes, have been detected in human TLE hippocampus (Ravizza et al., 2008a). Neuropathological examinations of brain specimens from patients affected by Rasmussen's encephalitis and active seizures revealed an increase in the expression of inflammation-related genes such as IL-1β and TNF-α and the presence of microglial nodules in close apposition to neurons (Baranzini et al., 2002). Moreover, cytotoxic lymphocytes have been described in the lesional tissue (Baranzini et al., 2002).

TSC is an autosomal dominant, multisystem disorder resulting from a mutation in the *tsc1* and *tsc2* genes. These two genes encode for two proteins (hamartin-tuberin) which form a complex with a negative regulator activity on mTOR. This protein is a kinase enzyme which is part of two signaling complexes (mTORC1 and mTORC2) that regulate cell metabolism, growth, proliferation and death and are involved in synaptic plasticity, neurogenesis, dendritic morphology and axonal sprouting (Ostendorf and Wong, 2015). Epilepsy is the most common neurologic symptom in TSC together with autism and cognitive disabilities. TSC patients are affected by brain lesions, named cortical tubers, characterized by the presence of dysmorphic neurons, giant cells and neuroinflammation with alterations in TNF-α and NF-kB expression, activation of the complement system and IL-1β signaling (Boer et al., 2008; Maldonado et al., 2003). Moreover, lymphocytes with T-cytotoxic phenotype, BBB leakage and high expression of cytokines were observed in the perivascular zone (Boer et al., 2008; Wong, 2008).

Focal cortical displasia (FCD) represent architectural malformations of the cerebral cortex, which are recognized as one of the most common causes of refractory epilepsy in children and young adults (Blumcke et al., 2009). FCD type II specimens demonstrated activation of complement and IL-1β signaling, induction of the chemokine CCL2, which regulates migration and infiltration of leukocytes, microglial reactivity as well as BBB breakdown (Iyer et al., 2010). There is also evidence of activation of TLR-mediated signaling (Zurolo et al., 2011).

Besides evaluating tissue samples with immunohistochemical analysis, measurements of pro-inflammatory mediators have been performed in biological fluids, such as csf and plasma (Gallentine et al., 2017; Lehtimaki et al., 2004; Virta et al., 2002; de Vries et al., 2016).

In a recent paper, Gallentine demonstrated that plasma IL-6 was significantly higher following febrile SE in children. The most important finding was the identification of the IL-1Ra/IL-6 ratio as a potential serologic biomarker of acute hippocampal injury following febrile SE and for the identification of patients at risk for developing mesial TLE (Diamond et al., 2014; Gallentine et al., 2017).

A role of inflammation in epilepsy is supported in the clinical setting by the anti-seizure activity of immunomodulatory drugs such as steroids and adrenocorticotropic hormone (Vezzani and Granata, 2005), particularly in pediatric epilepsies (Vezzani et al., 2015).

Specific anti-inflammatory drugs (Table 1.4.2), such as VX765 (ICE/Caspase-1 inhibitor) and Anakinra (IL-1R1 antagonist), demonstrated their therapeutic effects in experimental models and clinical signs of efficacy in humans (Bialer et al., 2013; Jyonouchi, 2016; Kenney-Jung et al., 2016).

In particular, **VX09-765-401** showed positive effect in a Phase 2 clinical trial for the treatment of pharmacoresistant partial epileptic patients (Phase II clinical trial number NCT01501383) (Bialer et al., 2013).

Anakinra was well-tolerated and effective in reducing pro-inflammatory cytokines in the csf and showed positive therapeutic effect in children affected by febrile infection-related epilepsy syndrome (FIRES) (Jyonouchi, 2016; Kenney-Jung et al., 2016).

Adalimumab, an inactivating antibody against TNF-α, determined a significant reduction in seizure frequency in patients affected by RE (Lagarde et al., 2016).

In an open-label interventional trial, patients with intractable epilepsies of different causes and types were treated with **cannabidiol** and the frequency of motor seizures was reduced by the 36,5% on average (Devinsky et al., 2016). Moreover, cannabidiol in 6/7 children affected by febrile infection-related epilepsy syndrome (FIRES) improved seizure frequency and duration

(Gofshteyn et al., 2017). The presence of cannabidiol CB2 receptors on immune cells suggest that this drug may act also as anti-inflammatory agent.

Moreover, a case report study in 2012 showed a marked reduction in seizure frequency during treatment with **minocycline**, a second-generation tetracycline antibiotic able to inhibit microglia activation, in a patient with severe symptomatic epilepsy due to an astrocytoma (Nowak et al., 2012).

Natalizumab, a humanized monoclonal antibody against alpha-4 (α4) integrin, probably reducing the entry of immune cells into the central nervous system, led to a dramatic reduction of tonic-clonic generalized seizures resistant to classical anti-epileptic drugs in a patient with multiple sclerosis (Sotgiu et al., 2010).

Table 1.4.2. *Target-specific anti-inflammatory treatments in epilepsy and neurological diseases with presentation of seizures: clinical studies.*

TARGET	DRUG	CLINICAL STUDY	REFERENCES
Caspase-1 (IL-1β, HMGB1)	VX09-765-401 (inhibitor)	Phase 2 (adult)	(Bialer et al., 2013)
IL-1R1	Anakinra (antagonist)	Case report FIRES (child); Case report (children, adolescent)	(Jyonouchi, 2016; Kenney-Jung et al., 2016)
TNF-α	Adalimumab (inactivating antibody)	Open pilot study RE (adult)	(Lagarde et al., 2016)
PPARγ/CBR/Adenosine/Nrf2	Cannabidiol (modulator)	Open label trial (intractable epilepsy); FIRES(children)	(Devinsky et al., 2016; Gofshteyn et al., 2017)
Microglia	Minocycline (inhibitor)	Case report astrocytoma (adult)	(Nowak et al., 2012)
Lymphocytes	Natalizumab (anti-α4 integrin antibody)	MS	(Sotgiu et al., 2010)

Abbreviations: FIRES, febrile infection related epilepsy syndrome; RE, Rasmussen's encephalitis; MS, multiple sclerosis.

1.4.3 Effect of anti-inflammatory treatments in experimental models of ictogenesis and epileptogenesis

The role of neuroinflammation in the pathogenesis of seizures and in epileptogenesis has been demonstrated by several experimental studies (Table 1.4.3). It is interesting to consider that two anti-convulsant drugs, such as **sodium valproate** (VPA) and **carbamazepine** (CBZ), have also anti-inflammatory actions. VPA inhibits the LPS-induced activation of NF-kB and the production of TNF-α and IL-6 in monocytes and glioma cells; CBZ inhibits the LPS-induced production of PGs in rat glial cells (Ichiyama et al., 2000; Matoth et al., 2000).

Glucocorticoids are immunosuppressive mediators with potent anti-inflammatory effects inhibiting the transcription of genes encoding for pro-inflammatory molecules by opposing the activation of AP-1 and NF-kB (Rhen and Cidlowski, 2005). However, a prolonged treatment with glucocorticoids after an epileptogenic injury may result in detrimental effect due to the large spectrum activity of these steroids. Data from Sapolski's lab showed that treatment with glucocorticoids may cause an increase in relative numbers of inflammatory cells and levels of the pro-inflammatory cytokines IL-1α, IL-1β and TNF-α (Dinkel et al., 2003). These data strongly question the traditional view of glucocorticoids being uniformly anti-inflammatory and could further explain how glucocorticoids worsen the outcome of neurological insults.

Thus, anti-inflammatory treatments aimed at blocking specific intracellular signaling pathways or specific mechanism may determine better therapeutic outcomes and reduce side effects (Table 1.4.3).

Minocycline was administered for 14 days following SE in rats injected with pilocarpine. The treatment inhibited microglia activation and IL-1β and TNF-α production induced by SE, without affecting astrocyte activation. In addition, minocycline prevented the neuronal cell loss following SE and reduced the frequency, duration and severity of seizures (Wang et al., 2015a). On the contrary, a sub-chronic minocycline administration initiated immediately following electrically-induced SE limited the spatial learning deficits and resulted in a selective

neuroprotective effect, but did not affect the development of spontaneous seizures in rats (Russmann et al., 2016). These data are controversial regarding the antiepileptogenic effect but suggest a disease-modifying impact of minocycline. Further exploration of the exact mechanisms underlying the beneficial effects is fundamental to define the use of this drug.

In 2012, Ma and colleagues showed that administration of **aspirin** (inhibitor of both COX-1 and -2) after pilocarpine-induced SE significantly reduced the frequency and duration of spontaneous recurrent seizures during the chronic epileptic phase (Ma et al., 2012). On the other hand, more than 10 years before, pre-treatment of rats with indomethacin, aspirin, nimesulide enhanced kainic acid induced seizures (Baik et al., 1999; Kunz and Oliw, 2001). These conflicting data on the effect of COX-1 and -2 inhibitors can be explained by considering that the various PGs can have both pro- and anti-inflammatory effects. In the last years, more specific and selective compounds have been tested in order to improve the therapeutic effect of COX-1 and -2 inhibition. Treatment with **selective COX-2 inhibitors**, such as Celecoxib, SC58236 and Parecoxib, administered after pilocarpine- or electrically-induced SE, indicated that COX-2 contributes to neuronal injury developing after SE, but inhibition of COX-2 does not appear to provide an effective strategy to modify epileptogenesis (Holtman et al., 2009; Jung et al., 2006; Polascheck et al., 2010).

Given the complexity of the inflammatory alterations following brain insults, it is more likely that a combined strategy targeting different pathways is needed for an efficient control of the pathological inflammatory cascade. To test this hypothesis, COX-2 inhibition was concomitantly performed with blockade of IL-1R1 with anakinra. This combined treatment showed better therapeutic effects, reducing the development of spontaneous seizures and limiting the extent of CA1 injury and mossy fiber sprouting (Kwon et al., 2013).

Another strategy to inhibit COX-2 pathway is **targeting EP1 or EP2 receptors** for PGE2, which is the major pro-inflammatory product of COX-2 in the brain (Liang et al., 2007). Ablation of EP1 or antagonism of EP2 after SE determines neuroprotection and reduced neuroinflammation and comorbidites (Chu et al., 2008; Rojas et al., 2015; Seeger et al., 2011).

Finally, Terrone and colleagues provided evidence supporting **MAGL** as a potential target for drug development in epilepsy. They reported significant reduction of the severity and duration of SE, of the consequent cell loss and an improvement of cognitive deficits (Terrone et al., 2017 *submitted*). One potential mechanism of the therapeutic effect of MAGL inhibition is the reduction of AA availability to COX-2, thus providing anti-inflammatory effects.

Intracerebroventricular injection of human recombinant **IL-1Ra** had powerful anti-convulsant activity on kainic acid- and electrically-induced seizures (De Simoni et al., 2000; Vezzani et al., 2000). Pre-treatment with IL-1Ra in the pilocarpine-induced SE model reduced the number of rats developing epilepsy and decreased the damage to the BBB (Marchi et al., 2009). Mice overexpressing the secreted form of IL-1Ra in astrocytes exhibit decreased seizure susceptibility (Vezzani et al., 2000). Importantly, seizures are dramatically reduced when blocking the endogenous IL-1β biosynthesis with the specific caspase-1 inhibitor, VX-765, or in mice with a null mutation of ICE/Caspase-1 (Ravizza et al., 2006a). A combined treatment with **anakinra** and **VX-765** performed during epileptogenesis starting 3 h after induction of electrical SE significantly reduced neuronal cell loss (Noe et al., 2013).

Inactivation of the biological effects of HMGB1 using **TLR4 antagonist**, such as BoxA (a fragment of HMGB1), *Rhodobacter sphaeroides* LPS and Cyanobacterial LPS (Cyp), resulted in a significant delay in the onset of seizures triggered in mice by kainic acid or bicuculline (Maroso et al., 2010).

IL-1β and HMGB1 pathways are concomitantly activated during epileptogenesis, thus likely requiring a rational drug combination for their effective blockade (Noe et al., 2013). Mice treated with a combination of **VX-765** and **Cyp** after the onset of epilepsy showed a reduction in seizure progression occuring in this model over 3 months post-SE leading to a 90% reduction of the frequency of spontaneous seizures (Iori et al., 2017b). The targeting of IL-1R1/TLR4 signaling has been attempted also by epigenetic intervention with miRNAs. In particular, **miRNA146a** is specifically associated with the modulation of IL-1R1/TLR4 signaling (Quinn and O'Neill, 2011) and is up-regulated in glial cells and to a lesser extent in neurons in human TLE

(Aronica et al., 2010). Intracerebroventricular injection of a synthetic mimic of miRNA146a significantly reduced neuronal excitability, preventing disease progression and dramatically reducing chronic seizure recurrence (Iori et al., 2017b).

In general, although anti-inflammatory treatments transiently given after the epileptogenic insult are unable to prevent the development of epilepsy, they significantly improve pathological outcomes (Table 1.4.3). Typically, the frequency and duration of seizures are reduced, and in several instances anti-inflammatory drugs afford neuroprotection, and improve comorbidities, in particular cognitive deficits. Interestingly, anti-inflammatory drug combinations may show improved therapeutic value as compared to drugs given alone (Vezzani et al., 2015). Since activation of inflammation is an endogenous homeostatic mechanism for tissue repair, the challenge is to counteract specific pathologic pathways with an appropriate therapeutic window of intervention.

Table 1.4.3. *List of treatments administered after SE or after disease onset showing disease-modifying effects* (Terrone et al., 2016).

TREATMENT (ACTION)	MOLECULAR TARGETS	REDUCTION IN
Celecoxib (enzyme inhibitor)	COX-2	SRS frequency/duration, cell loss (Jung et al., 2006); pharmacoresistance (Schlichtiger et al., 2010)
Parecoxib (enzyme inhibitor)	COX-2	Cell loss (Polascheck et al., 2010)
Aspirin (enzyme inhibitor)	COX-1/2	SRS frequency/duration; cell loss (Ma et al., 2012)
α4-integrin-specific Ab (receptor antagonist)	Adhesion molecules; brain vessels	SRS frequency; anxiety (Fabene et al., 2008)
Fingolimod (receptor antagonist)	S1P receptor	SRS frequency/duration/severity; cell loss (Gao et al., 2012)
Minocyclin (microglia inhibitor)	Cytokines	Cognitive deficits; cell loss (Russmann et al., 2016); SRS frequency/duration/severity; cell loss (Wang et al., 2015a)
Anakinra + VX-765 (receptor antagonist + enzyme inhibitor)	IL-1R1 + ICE/caspase-1	Cell loss (Noe et al., 2013)
Anakinra + COX-2 (receptor antagonist + enzyme inhibitor)	IL-1R1 + COX-2	SRS frequency; cell loss (Kwon et al., 2013)
TLR4 + VX-765* (receptor antagonist + enzyme inhibitor)	TLR4 + ICE/caspase-1	SRS frequency & their progression; cognitive deficits; cell loss (Iori et al., 2017b)
miRNA146a (epigenetic antagonism)	IL-1R1 + TLR4 signaling	SRS frequency & their progression; cognitive deficits; cell loss (Iori et al., 2017b)
Nrf2 gene therapy* (transcription factor)	Oxidative stress	SRS frequency/duration; cell loss (Mazzuferi et al., 2013)
Erythropoietin (agonist)	Erythropoietin receptor	Cell loss (Jiang et al., 2015)
PGE2 (antagonist)	EP2 receptor	SRS frequency/duration; cell loss (Chu et al., 2008); cognitive deficits; cell loss (Seeger et al., 2011)
MAGL (inhibitor)	MAGL enzyme	SE severity/duration; cell loss and cognitive deficits (Terrone et al., 2017 *submitted*)

Minozac, an inhibitor of astrocytes activation, has been shown to raise seizure threshold and improve comorbidites in a model of post-traumatic epilepsy (Chrzaszcz et al., 2010). SRS, spontaneous recurrent seizures; S1P, sphingosine-1 phosphate. (*) Treatments administered after disease onset.

1.5 PRO-RESOLVING MECHANISMS OF NEUROINFLAMMATION

Endogenous mechanisms for rapid and effective resolution of the inflammatory process are pivotal to prevent tissue dysfunction. It is now clear that resolution is an active mechanism triggered by the same cells responsible for the inflammatory response (Serhan, 2007). At the beginning of the immune response, pro-inflammatory mediators are predominantly localized in the injured site. Their up-regulation is subsequently counterbalanced by anti-inflammatory and pro-resolving signals generated to attenuate and limit inflammation (Figure 1.5.1).

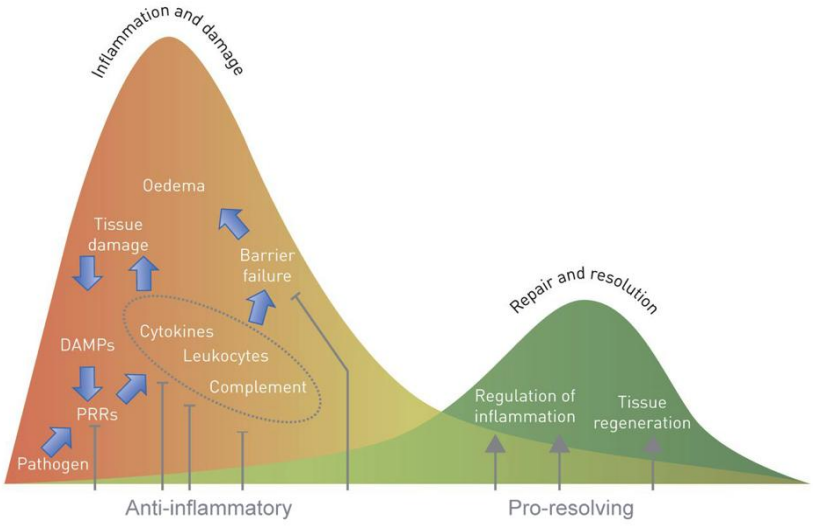


Figure 1.5.1. Time course of inflammation and resolution. *Subsequent to ligand binding, pathogen recognition receptors (PRRs) initiate the inflammatory response (red area), including cytokine and chemokine production, complement activation and leukocytes recruitment (mainly in inflammatory diseases of the periphery). Immune overactivation may lead to tissue damage and vascular barrier breakdown. From the beginning of the inflammatory response, resolution processes are initiated (green area) to balance damage and healing of the tissue, ideally resulting in restoration of the original condition (modified by Müller-Redetzky et al., 2015).*

Various active mechanisms modulate the inflammatory response: (a) proteins competing with cytokines for the binding to their receptors, such as IL-1Ra; (b) decoy receptors able to recognize and bind efficiently specific growth factors or cytokines, but not structurally able to activate the intracellular signaling pathway; (c) molecules with anti-inflammatory properties (such as IL-10 and TGF-β). (d) Glucocorticoids play a pivotal role in terminating the inflammatory response. Activation of their specific intracellular receptors determines the

down-regulation of NF-kB and AP-1, thus switching off the inflammatory response. (e) Another endogenous mechanism that limits the inflammatory response is the efferent activity of the vagus nerve that inhibits the release of pro-inflammatory molecules by resident macrophages (Tracey, 2002, 2007). (f) An important role in triggering resolution is played by neutrophils and monocytes/macrophages activating a specific pro-resolving transcriptional program (Serhan, 2007). These cells produce specialized pro-resolving mediators (SPMs), lipid (lipoxins, resolvins, protectins) or peptide (IL-1Ra, annexin A1) molecules able to turn off the inflammatory response and trigger resolution. These molecules are produced at the inflammatory site, elicit very rapid effects and are rapidly inactivated (Serhan et al., 2008). They exert their pro-resolving action on target cells by interacting with specific G-protein-coupled receptors activating a series of intracellular signaling pathways resulting finally in an anti-inflammatory effect. These SPMs are not immunosuppressive, but activate specific pathways that restore tissue homeostasis (Serhan et al., 2008) without inhibiting the acute phase of the immune response, essential step for triggering resolution itself. Resolution has been extensively studied in inflammatory diseases of the periphery. In general, peripheral activation of resolution determines different effects on multiple target cells:

- provides signals that selectively block the infiltration of polymorphonucleates;
- stimulates monocyte recruitment and promotes the switch to an anti-inflammatory phenotype;
- activates phagocytosis of microorganisms and apoptotic immune cells;
- increases phagocytic elimination of lymphocytes which have terminated their task.

In the context of neuroinflammation, SPMs inhibit the synthesis of pro-inflammatory molecules produced by glial cells and promote an anti-inflammatory phenotype (Serhan, 2007; Serhan and Chiang, 2008). In the CNS, however, resolution is still poorly investigated.

1.5.1 Lipid mediators of resolution

Resolution of inflammation is driven by the production of some lipid mediators. In the early phases, eicosanoids such as PGs and leukotrienes amplify the inflammatory response against tissue injury. In particular, PGE2 and PGD2 are produced during the acute phase of the immune response, starting from AA through the activity of COX-1 and COX-2 enzymes. Thereafter, these first-stage agonists are able to initiate resolution. They induce transcription of key enzymes, named lipoxygenase (LOX), required for the generation of other classes of eicosanoids. In particular, induction of LOX enzymes results in the production of pro-resolving lipoxins from AA and of resolvins, protectins and maresins from omega-3 polyunsaturated fatty acids (ω-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Serhan et al., 2008). The switch from classic prostaglandins and leukotrienes, to the production of immunoresolvents is an active process named "lipid mediators class switching" and is an essential step for triggering the correct resolution program (Figure 1.5.2, 1.5.3) (Levy et al., 2001).

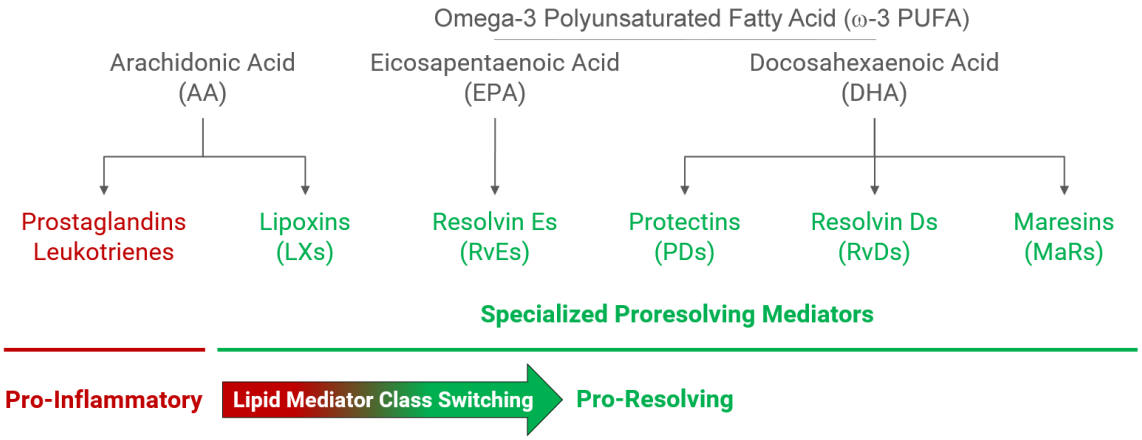


Figure 1.5.2. Lipid mediator class switching *(http://thetispharma.com/cms2/approach/lipid-biology/).*

Importantly, inhibition of COX-2 delays resolution because prostaglandins are essential initiators of lipid-mediator class switching, as shown in animal disease models *in vivo* (Chan and Moore, 2010; Levy et al., 2001).

1.5.1.1 Lipoxins

Lipoxin (LX) was the first lipid mediator with anti-inflammatory and pro-resolving action to be identified. As previously mentioned, lipoxins as well as prostaglandins and leukotrienes, are produced from AA by the action of LOX (Figure 1.5.3). The generation of lipoxins is a transcellular mechanism because it is driven by a combination of different forms of LOX (LOX5, LOX12 and LOX15), which are expressed in specific cell population (leukocytes, neutrophils or blood vessels). In Alzheimer's disease expression of LOX was shown in astrocytes and microglial cells (Wang et al., 2015b).

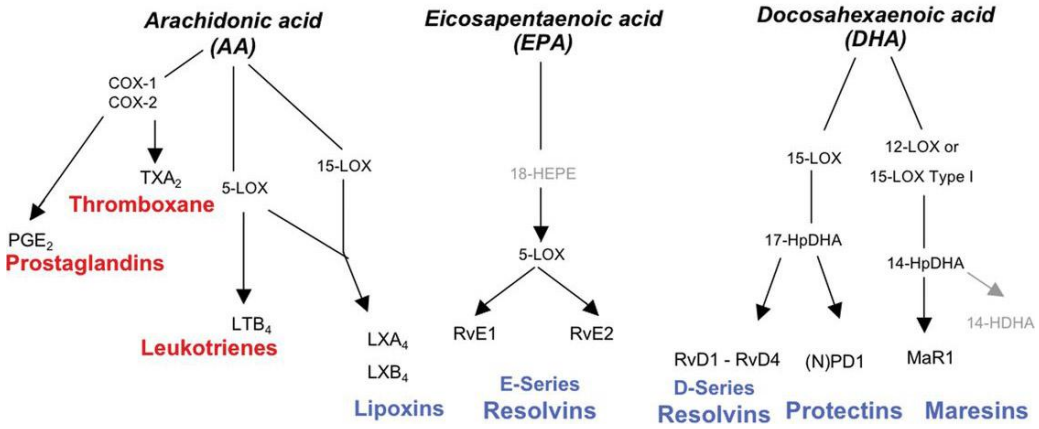


Figure 1.5.3. Schematic drawing of eicosanoids and specialized pro-resolving mediators (SPMs) generation. *Pro-inflammatory AA-derived eicosanoids are in red. Pro-resolving molecules are in blue: AA-derived lipoxins, EPA-derived E-series resolvins, DHA-derived D-series resolvins, protectins and maresins. A key role in the lipid mediator class switching is played by COX and LOX enzymes (Fredman and Serhan, 2011).*

Lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) represent the first members of the family (Figure 1.5.3). Their biological activity was characterized both *in vitro* and *in vivo* (Serhan and Chiang, 2008). These eicosanoids are highly conserved in all species, from fish to mammals (Serhan and Savill, 2005). They are produced in pico- or nano-grams amount predominantly from innate immune cells and act in autocrine and paracrine way.

In the presence of aspirin, cells that express COX-2, including endothelial cells, epithelial cells, macrophages and neutrophils, are involved in the production of aspirin-triggered lipoxin (ATL) or 15-epi-LXA_4, more stable and powerful than LXA_4 (Figure 1.5.4).

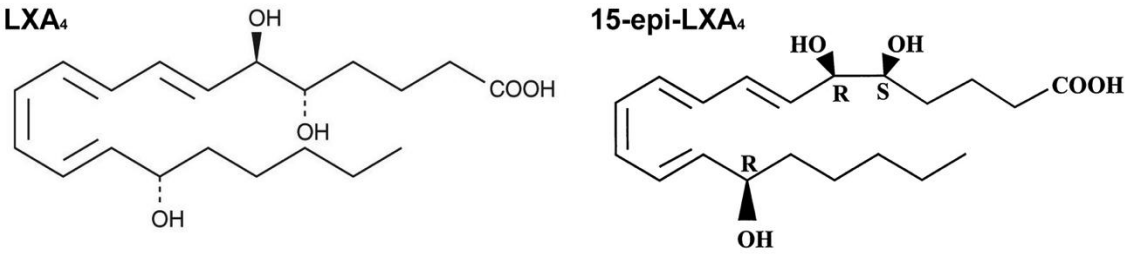


Figure 1.5.4. Chemical structures of LXA_4 and 15-epi-LXA_4 *(modified by Serhan, 2010).*

In particular, aspirin acetylates COX-2, blocking its ability to produce prostaglandins, and then ATL is synthesized by the action of LOX5. Once generated, lipoxins are rapidly metabolized and enzymatically inactivated by 15-hydroxyprolyteglandin dehydrogenase and consequently have a short half-life (Wu et al., 2011). To overcome this disadvantageous feature of lipoxins in view of possible clinical application, a synthetic analogue was developed by adding a methyl ester group to LXA_4 (LXA_4ME). This synthetic molecule is chemically more stable and preserve the anti-inflammatory and anti-oxidant activity of the native molecule (Jin et al., 2012).

The anti-inflammatory and pro-resolving activity of lipoxin was characterized and confirmed by experimental studies in animal models of peripheral inflammatory diseases (Jin et al., 2012; Svensson et al., 2007; Ye et al., 2010). Lipoxins inhibit chemotaxis, cytokine production, reactive oxygen species (ROS) formation and NF-kB activation (Serhan and Savill, 2005). Expression of LOX, LXA_4 and its specific receptor has also been identified in the CNS (Wang et al., 2015b). Furthermore, it has been shown that LXA_4 is able to reduce levels of ROS in activated microglial cells (Wu et al., 2012b), to inhibit the expression of the cytokine IL-8 in astrocytoma cells (Decker et al., 2009) and to reduce levels of β-amyloid and enhance cognitive ability in transgenic mice affected by Alzheimer's disease (Medeiros et al., 2013).

1.5.1.2 Resolvins

For a long time, beneficial actions of ω-3 PUFAs, administered daily at high doses, have been observed in many inflammatory pathologies (Simopoulos, 2002).

Resolvins derive from EPA or DHA with two different structural forms, the E-series and D-series, respectively (Figure 1.5.3). RvE1 was the first resolvin identified in resolving inflammatory exudates by liquid chromatography-ultaviolet-tandem mass-spectrometry (Serhan et al., 2000). The mature form of RvE1 is produced after different steps starting from EPA and the enzyme responsible for the last step is LOX (Figure 1.5.3, 1.5.5). Using the same biosynthetic pathway of RvE1, other members of E-series family are produced, from RvE2 to RvE6.

In addition to resolvins of the E-series, other lipid mediators have been identified in inflammatory exudates: the D-series resolvins (RvDs) derived from DHA. RvDs display a number of potent anti-inflammatory actions and they are of particular interest, because DHA is the most abundant lipid in synapses, retina and the brain. Endogenous DHA is converted *in vivo* by LOX15 to the 17S-hydroxy-containing series of four resolvins, known as RvD1-4 (Figure 1.5.3, 1.5.5). These resolvins reduced levels of pro-inflammatory transcripts in microglia, including the one encoding for IL-1β (Serhan et al., 2008). Aspirin-triggered analogs as well as more stable synthetic analogs have been synthesized also for these lipid mediators.

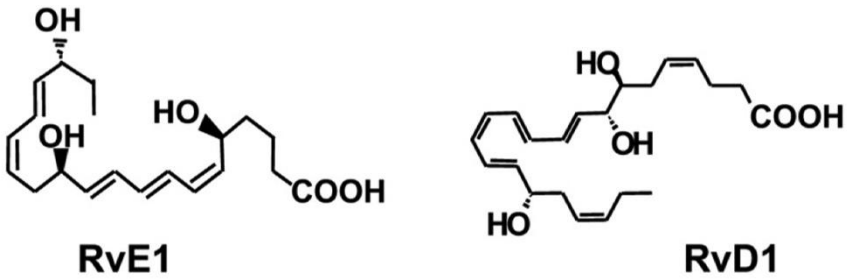


Figure 1.5.5. Chemical structures of RvE1 and RvD1 *(modified by Serhan, 2010).*

1.5.1.3 Protectins

Protectins are the last class of lipid mediators identified and derived from DHA, distinguished by the presence of a conjugated triene double bond. Protectin biosynthesis is mediated by LOX15 which converts DHA into an oxidized intermediate, rapidly converted to give the bioactive final compound: 10,17S-docosectrin, known as Protectin D1 (PD1; Figure 1.5.3, 1.5.6). When produced in the brain it is commonly termed neuroprotectin (NPD1), the prefix neuro is added to describe its brain origin (Serhan, 2007; 2008). This lipid mediator is one of the most powerful autacoids known today: down-regulates COX-2, thus inhibiting the production of pro-inflammatory eicosanoids derived from AA; reduces NF-kB activation; reduces pro-apoptotic regulators and increases anti-apoptotic regulators; reduces neuronal cell death through reduction of caspase activation (Bazan, 2005; Bazan et al., 2005).

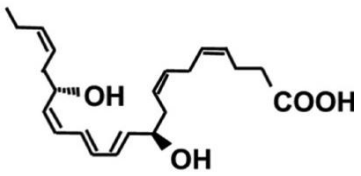


Figure 1.5.6. Chemical structure of PD1 *(modified by Serhan, 2010).*

1.5.2 Peptide mediators of resolution

Besides the lipid mediators, SPMs include some peptides able to mediate the resolution of the inflammatory response, such as IL-1Ra and Annexin A1.

1.5.2.1 IL-1Ra

IL-1Ra recognizes and binds IL-1R1 with high affinity without activating any intracellular response (Dinarello, 1996). Most importantly, in order to be effective the antagonist levels should exceed at least 100-1000 times IL-1β levels (see Section 1.4.1.1) (De Simoni et al., 2000). However, during epileptic seizures, insufficient levels of IL-1Ra are produced. Implementing

these levels reduce seizure number, delay their occurrence and protect neurons from damage (Maroso et al., 2011a; Ravizza and Vezzani, 2006; Vezzani et al., 2000, 2011b).

1.5.2.2 Annexin A1

The most well-known pro-resolving peptide mediator is Annexin A1, a monomeric, anphipatic protein consisting of 346 amino acids. Annexin A1, also called lipocortin, is the first of 13 members of the annexin family, characterized by common structural features, such as the Ca^{2+} binding site, which enables Annexin A1 to bind acidic phospholipids of cellular membrane (Figure 1.5.7) (Gerke and Moss, 2002; Gerke et al., 2005). In addition to mediating membrane binding, Ca^{2+} can also induce a conformational change that leads to the exposure of the bioactive N-terminal domain. Indeed, studies on the anti-inflammatory activity of Annexin A1 revealed not only that the different functions of the protein lie within the unique N-terminus, but also that synthetic peptides from the N-terminal domain, such as peptide Ac2-26, may mimic the pharmacological property of the whole protein, specifically binding to formyl peptide receptors (FPRs; Figure 1.5.7) (Gerke et al., 2005; Perretti and Dalli, 2009).

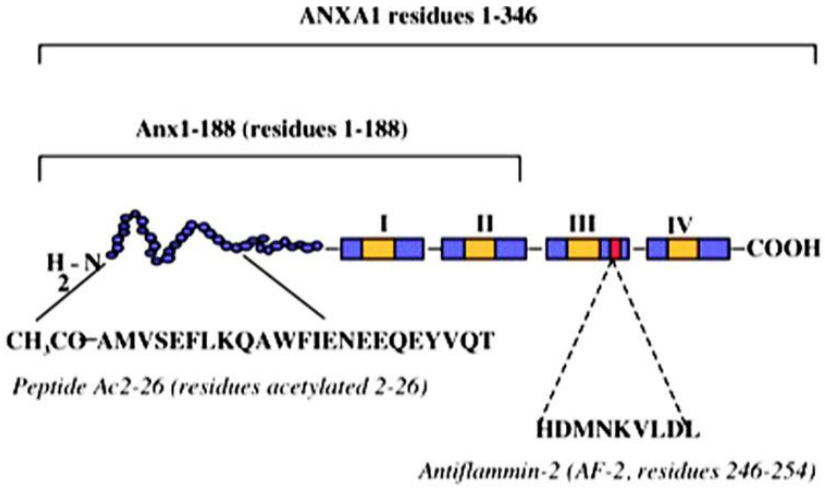


Figure 1.5.7. Peptide structure of Annexin A1 *(Gavins and Hickey, 2012).*

Following cell activation, Annexin A1 is immediately secreted by a cell-specific mechanism. The absence of a peptide sequence that acts as signal for secretion prevents the release of the protein through the canonical pathway. For example, in macrophages the release of Annexin A1 seems to be mediated by the activity of an ATP-Binding Cassette family carrier (Perretti et al., 1996).

The modulatory action of Annexin A1 on inflammation is closely related to the action of glucocorticoids (Blackwell et al., 1980; Flower, 1988). They modulate levels of anti-inflammatory and pro-resolving mediators, including Annexin A1, through genomic or non-genomic mechanisms, respectively modulating gene transcription or the availability of the protein (D'Acquisto et al., 2008).

Dexamethasone and other steroids trigger mobilization of Annexin A1 from the plasma membrane, without requiring new synthesis of the protein (Perretti and Dalli, 2009). This effect is rapid (about 5-10 min) and lasts for 30-90 min, or until complete depletion of the intracellular source of the peptide mediator. Alternatively, steroid hormones and their synthetic analogues mediate a genomic regulation, inducing the transcription of Annexin A1 gene. This mechanism is slower, starts 2-4 hours after steroid stimulation and lasts for 18-24 h (Perretti and D'Acquisto, 2009). In both cases, the effects mediated by Annexin A1 in response to glucocorticoids, are anti-inflammatory and immunosuppressive, thus enhancing the resolution of the acute inflammatory response.

In addition, dexamethasone treatment is able to inhibit the expression of COX-2 and of the inducible form of nitric oxide synthase (iNOS). The resulting reduction in PGE2 and nitric oxide (NO) production could contribute to the neuroprotective effect of lipocortin. Finally, Annexin A1 mRNA levels are significantly increased in microglial cells activated following brain injury, and the protein is secreted by the same activated cells (Parente and Solito, 2004).

1.5.3 Pro-resolving receptors: ALXR and ChemR23

Pro-resolving mediators described so far, elicit their action by activating specific G-protein coupled receptors (GPCRs). These receptors are characterized by seven putative transmembrane domains with an N-terminus on the extracellular side of the membrane and a C-terminus on the intracellular side. Four different receptors for these mediators have been identified: the LXA_4 (ALXR) receptor, the chemerin receptor (ChemR23), the GPR-1 and GPR-32 orphan receptors (Table 1.5.1). In this thesis, the attention is more focused on ALXR and ChemR23 since they are the best characterized.

Table 1.5.1. *Pro-resolving mediators and their respective G protein coupled receptors (GPCRs).*

Ligands	GPCRs
RvD1 Lipoxin (LX) A_4 Annexin A1	ALXR (i.e. Lipoxin A_4 receptor/FPRL1/FPRL2)
Resolvin (Rv) E1 Chemerin	ChemR23
Chemerin	GPR-1
RvD1 LXA_4	GPR-32

1.5.3.1 ALXR

LXA_4 receptor (ALXR) is also known as FPR2, since it was initially identified and characterized as "receptor for formyl peptides 2". It is part of a family with 2 other members: FPR1 and FPR3. These receptors are coupled to G inhibitory protein and their function was initially attributed to the recognition of bacterial peptides. ALXR is expressed by several cell populations such as neutrophils, macrophages, monocytes, endothelial and epithelial cells in peripheral organs (Perretti and D'Acquisto, 2009). It can be recognized and bound by various agonists, peptides and lipids with different molecular structures. Among the identified ligands there are: the serum amyloid A, with a potent chemoattractant activity and able to determine an increased production of the pro-inflammatory cytokine IL-8 by neutrophils; a portion of the chemokine

CCL23; β-amyloid 42 and a protein from the bacterium Helicobacter pylori (Waechter et al., 2012). All these ligands, interacting with ALXR on target cells, mediate pro-inflammatory effects. In contrast, the activation of this receptor mediated by Annexin A1 and LXA_4 results in anti-inflammatory effects (Figure 1.5.8) (Fernandez-Patron and Filep, 2012).

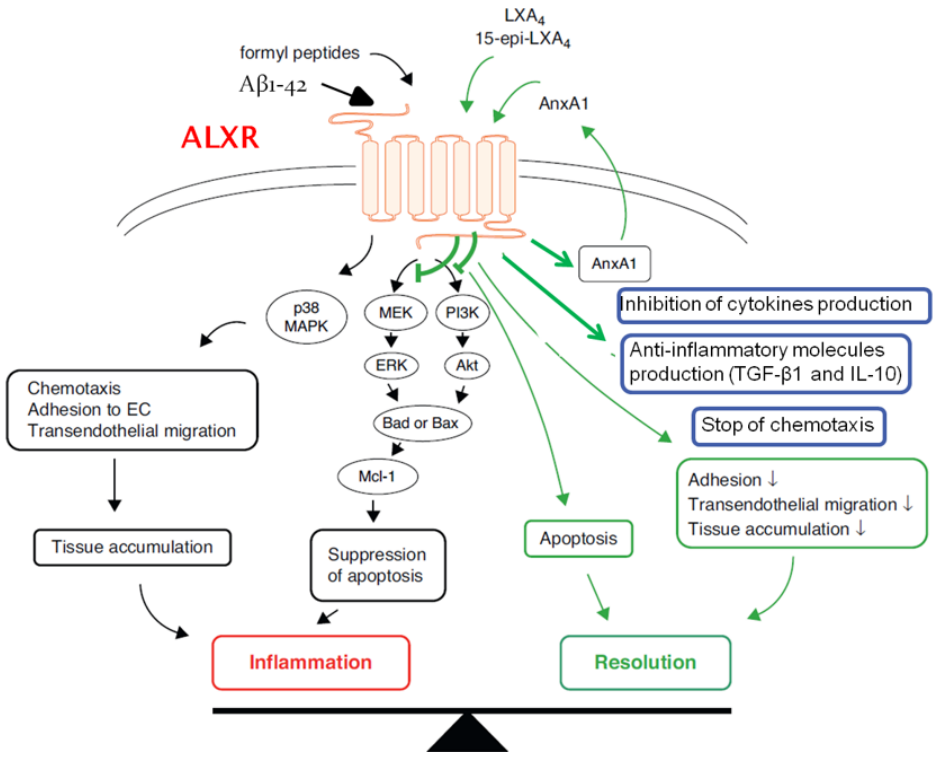


Figure 1.5.8. Schematic drawing of intracellular signaling cascade and biological effects mediated by the activation of ALXR *by pro-inflammatory (right) and pro-resolving (left) ligands (modified by Fernandez-Patron and Filep, 2012).*

The dual nature of the intracellular signaling pathways induced by ALXR activation suggests that receptor expression is finely regulated in relation to the time and cell population in which it is expressed. It has been shown that FPR2 gene expression is regulated by glucocorticoid. In addition to increasing the expression of Annexin A1, dexamethasone and other synthetic analogs induce ALXR expression at 4-6 h after treatment (Perretti and D'Acquisto, 2009).

1.5.3.2 ChemR23

The first identified ligand of ChemR23 was chemerin, the protein which gave the name to the receptor. Chemerin has been recently described as an adipokine: it is synthesized by adipose tissue and it is associated with obesity, insulin resistance and metabolic syndromes. This molecule is a powerful chemoattractant, but during resolution exerts a number of anti-inflammatory actions (Mariani and Roncucci, 2015). Besides ChemR23, chemerin binds chemokine receptor-like 2 (CCRL2) and the orphan receptor GPR1. Following binding to CCRL2, this adipokine acts as pro-inflammatory cytokine in peripheral tissues (Mattern et al., 2014). In addition to chemerin, the lipid mediator RvE1 activates ChemR23 (Arita et al., 2005) and reduces edema formation, leukocyte infiltration, cytokine and chemokine expression (Xu et al., 2010).

ChemR23 has a high sequence homology with the receptors for complement system components, such as C3a and C5a, as well as with receptors for formyl peptides, including ALXR (Mariani and Roncucci, 2015). Through the recruitment of inhibitory G protein, the activation of ChemR23 results in intracellular Ca^{2+} increase, inhibition of both cAMP production and MAPK activation. It also triggers the PI3K/Akt signaling with promotion of tissue repair and neurite outgrowth (Mariani and Roncucci, 2015).

The cell population and the phase of the inflammatory process in which ChemR23 is expressed determine its pro-inflammatory and pro-resolving biological effects (Herová et al., 2015).

1.5.4 Therapeutic effects of pro-resolving mediators in experimental models of neurological diseases

Pro-resolving mediators represent a promising therapeutic approach for disease characterized by chronic inflammation. Thanks to their activity as "stop signals" of the acute inflammatory response, they could resolve inflammation and reduce or prevent tissue damage. The first evidence of the efficacy of these molecules was obtained by experimental studies conducted in inflammatory diseases affecting peripheral organs. In various animal models reproducing pathological conditions with an inflammatory component such as ocular, kidney, cardiovascular, liver, pulmonary and gastrointestinal diseases, lipoxins, resolvins and protectins, as well as their aspirin-triggered analogues demonstrated a powerful anti-inflammatory activity (Serhan et al., 2014).

The therapeutic effects of SPMs in experimental models of epileptogenesis are very scarse (see Section 1.5.5), therefore, in the next paragraphs is reported evidence of pro-resolving mediator effects in other experimental models of PNS and CNS pathologies.

1.5.4.1 Neuropathic pain

Neuropathic pain is a pathological condition with an important inflammatory component. In an animal model of neuropathic pain generated by intra-plantar formalin administration, treatment with a synthetic stable analogue of RvE1 reduced hyperalgesia. Xu and colleagues demonstrated that the therapeutic effects of RvE1 were mediated by the activation of ChemR23 (Xu et al., 2010).

In addition, intrathecal injection of LXA_4 or its aspirin-triggered form, reduced hyperalgesia, microglia activation, TNF-α levels in different models of neuropathic pain (Martini et al., 2016; Svensson et al., 2007).

In a mouse model of spinal injury induced by the constriction of the sciatic nerve, local treatment with PD1 at the time of the surgery prevented mechanical allodynia and the onset of

neuropathic pain as well as promoted spinal neuroprotection. Moreover, PD1 prevented nerve injury-induced microglial and astrocytic activation, phosphorylation of p38 and ERK, and the associated up-regulation of IL-1β and CCL2 (Xu et al., 2013).

Finally, the administration of Ac2-50, a peptide derived from the N-terminal portion of Annexin A1, reduced mouse inflammatory pain. The antinociceptive effect of Ac2-50 was explained by its ability to inhibit the molecular cascade linked to PGE2 and to reduce the inflammatory process (Ayoub et al., 2008).

1.5.4.2 Neurotrauma

In models of neurotrauma, a pathological event that evokes a neuroinflammatory phenomenon and increases the risk of developing epilepsy, intracerebroventricular injection of LXA_4 reduced BBB damage, edema, and volume of brain injury. The therapeutic effect was associated with a reduction of mRNA and protein levels of TNF-α, IL-1β and IL-6, while ALXR levels increased in resident astrocytes in the lesioned cortex (Luo et al., 2013).

In a model of traumatic brain injury in mice, RvE1 (ChemR23 receptor ligand) and aspirin-triggered analogue of RvD1 (AT-RvD1, ALXR receptor ligand) were administered intraperitoneally for seven days beginning 3 days prior to trauma. AT-RvD1, but not RvE1, reduced cognitive and motor deficits associated with trauma (Harrison et al., 2015).

1.5.4.3 Ischemia

Acute inflammatory response contributes to ischemic brain injury. To this end, several promising studies on the neuroprotective effects of SPMs have been conducted in this pathological condition.

In experimental models of ischemia in rats, intracerebroventricular injections of LXA_4 (Wu et al., 2012a) or of its more stable methylated form LXA_4ME (Ye et al., 2010) or intravenous administration of its analogue BML111 (Hawkins et al., 2014) reduced the infarcted area, the

number of apoptotic neurons, glial activation and recruitment of neutrophils. In addition, the treatment significantly reduced TNF-α and IL-1β protein levels and increased the production of anti-inflammatory cytokines, IL-10 and TGF-β (Ye et al., 2010).

Annexin A1, another ALXR receptor ligand, showed neuroprotective action in experimental models of ischemia (Gavins et al., 2007). In this study, the mRNA and protein levels of Annexin A1 were down-regulated and inversely correlated to the ischemic area. Furthermore, KO mice for the lipocortin gene exhibit greater susceptibility to brain damage following reperfusion, increased mortality, and greater pro-inflammatory cytokine production. Ac2-50 systemic injection during reperfusion significantly reduced the damaged area (Gavins et al., 2007).

PD1 continuous intravenous administration for 48 h after ischemic reperfusion in mice reduced the cerebral infiltration of polymorphonuclear leukocytes and the expression of COX-2 and NF-kB. The final outcome was a reduction in the infarctuated area in hippocampus and cortex (Marcheselli et al., 2003).

1.5.4.4 Alzheimer's disease (AD)

Experimental studies conducted in murine AD models demonstrated reduction of neuroinflammation, neurodegeneration and improvement of cognitive deficits after treatment with lipoxin analogs, such as LXA_4ME (infused intracerebroventricularly) (Wu et al., 2011) and ATL (injected subcutaneously for 8 weeks) (Medeiros et al., 2013).

Microglial and astrocytic activation, due to the presence of β-amyloid oligomers, was reduced by PD1 which decreased pro-inflammatory cytokines and inhibited caspase-3, thus favoring the expression of anti-apoptotic proteins (Zhao et al., 2011).

RvD1 added to macrophages of patients with AD *in vitro* reduced the pro-inflammatory phenotype and enhanced phagocytosis of β-amyloid (Mizwicki et al., 2013).

1.5.5 Therapeutic effects of pro-resolving mediators in hyperexcitability and seizures

The role of pro-resolving lipids in hyperexcitability has been studied mostly for DHA and its derivative NPD1. Under resting conditions, unesterified DHA is present in very low amounts in the brain and is associated to membrane phospholipids (Bazan, 2003). Seizures induce phospholipases activity and as a consequence, esterified DHA is released from membrane phospholipids (Bazan, 2003).

DHA may elicit anti-seizure activity through modulation of ion channels and neurotransmitter receptors, as well as regulation of synaptic plasticity (Champeil-Potokar et al., 2006; Young et al., 2000). A first indication of DHA role in excitability, was reported by intracellular recording of neurons in the *stratum pyramidale* of rat hippocampal slices where DHA inhibited the repetitive firing of action potentials (Young et al., 2000).

Recent evidence suggests that DHA improves neurological outcomes in models of epilepsy (Musto et al., 2011; Young et al., 2000) and is neuroprotective (Belayev et al., 2009).

NPD1 delivered intracerebroventricularly by osmotic minipumps during kindling epileptogenesis limited progression of seizure severity and hippocampal hyperexcitability evoked in the hippocampus (Musto et al., 2011). In a following paper the same group administered NPD1 intraperitoneally for 5 days after pilocarpine induced SE. The treatment reduced the number and duration of spontaneous seizures evaluated 3 weeks after SE and protected hippocampal dendritic spines during epileptogenesis (Musto et al., 2015).

1.5.6 Clinical evidence of resolution mechanisms in human pathologies

Since resolvins and protectins were identified in mouse exudates, it was essential to establish their biosynthesis also in human blood and tissue. SPMs were firstly identified in human blood, placenta and milk by liquid chromatography-tandem mass spectrometry (LC/MS-MS) (Serhan et al., 2014). Then, the availability of SPMs provided opportunities for developing more rigorous assessment techniques. In 2014, using targeted LC/MS-MS-based lipid mediators metabololipidomics, SPM pathways was quantified in human peripheral blood (plasma and serum) and lymphoid organs. RvD1, RvD2, RvD3 and NPD1/PD1 were identified in amounts within their bioactive ranges for their pro-resolving, organ protective, and tissue regeneration functions (Colas et al., 2014).

Some studies investigated resolution mechanisms in AD (Lukiw et al., 2005; Mizwicki et al., 2013; Wang et al., 2015b), multiple sclerosis (Prüss et al., 2013) and rheumatoid arthritis (Giera et al., 2012) affected patients.

DHA and PD1 were reduced in AD hippocampal cornu ammonis (CA) region 1, but not in the thalamus or occipital lobes from the same brains. The expression of key enzymes in PD1 biosynthesis, cytosolic PLA2 and LOX15, was altered in AD hippocampus (Lukiw et al., 2005).

Wang and colleagues reported the presence of pro-resolving mediators and their receptors in the human brain (Wang et al., 2015b). ELISA and LC/MS-MS were used to quantify levels of LXA_4 and RvD1 in csf and hippocampus of AD patients as compared to controls. Data demonstrated a reduction of LXA_4, but not of RvD1, in affected patients, while levels of prostaglandins were higher in AD than in control, supporting a pro-inflammatory state. The decreased levels of LXA_4 were not due to lower levels of its biosynthetic enzyme LOX15, since its staining was increased in glial cells in the hippocampus. The pro-resolving receptors, ALXR and ChemR23, were increased in astrocytes and microglia in AD hippocampus as compared to control tissue. The increased levels of ALXR may indicate a compensatory reaction to reduced levels of its ligand, LXA_4 (Wang et al., 2015b). When tissue levels of pro-resolving mediators are

reduced, ALXR and ChemR23 may mediate neurotoxic effect of pro-inflammatory molecules such as β-amyloid (Le et al., 2001).

In recent years, several clinical trials with synthetic analogue of lipoxins, resolvins and protectins have been performed with very promising results. Specifically, RX-10045, a prodrug of RvE1 has successfully passed a phase II clinical trial for topic treatment of dry eye syndrome (http://www.auventx.com/auven/products/rx10045.php). PD1 is in clinical development for neurodegenerative diseases (http://www.anidapharma.com/lead-molecule.html) and topical administration of 15(R/S)-methyl-LXA_4 relieved severity and improved quality of life of infants with eczema in a double-blinded trial (Wu et al., 2013). Most ω-3 fatty acids used in clinical trials or experimental designs related to epileptic disorders include EPA and DHA, which are administered along with anti-epileptic drugs (Taha et al., 2010). EPA/DHA supplementation reduces the number of rescue-medication doses required during the maintenance phase. Today, the recommended treatment is for at least 6 months at high doses. No side effects have been described till now and another advantage of omega-3 PUFAs is that they protect against cardiovascular diseases (Yuen et al., 2005). A n-3 PUFAs-enriched diet would serve as an adjunct to the drugs in patients with drug-resistant seizures or in patients with first presentation of seizures or exposed to epileptogenic injuries.

If a chronic inflammatory component results from failed resolution mechanisms then rescue of these mechanisms may represent a strategy for modulating inflammation in order to attain therapeutic effects.

1.5.7 Experimental and clinical evidence of inefficient control of brain inflammation in epilepsy

There is evidence that neuroinflammation is not efficiently controlled in human and experimental epilepsy therefore contributing to brain dysfunction.

The extent of expression of IL-1β largely anticipates that of IL-1Ra in animal models (De Simoni et al., 2000). Moreover, in epilepsy-associated malformations of cortical development IL-1β and its receptor IL-1R1 were highly expressed whereas IL-Ra was expressed to a lesser extent (Ravizza et al., 2006b). These findings show a relative deficient mechanisms (IL-1Ra) apt to inactivate IL-1β actions.

Proteins involved in the inhibitory control of the complement system, such as Daf1, CD59 and Crry, are induced in the hippocampus during seizures to a lower extent than those of the complement activating factors (Aronica et al., 2007).

The activating transcriptional factor 3 (ATF-3) that negatively controls the induction of TLR4 is expressed in the brain at lower levels in patients with more frequent seizures, while TLR4 levels positively correlate with the frequency of seizures (Pernhorst et al., 2013).

The membrane glycoproteins CD47 and CD200, mediate inhibitory signals for microglia/macrophage activation. These proteins are downregulated in epileptogenic lesions where chronic neuroinflammation has been established. Up-regulation or activation of CD47 and CD200 may have therapeutic potential for controlling neuroinflammation in patients with epileptogenic lesions (Sun et al., 2016).

Altogether these findings strongly suggest that brain inflammation is inefficiently controlled in epilepsy.

CHAPTER 2 - AIMS OF THE THESIS

The hypothesis underlying this thesis is that the homeostatic resolution response controlling neuroinflammation is not efficiently activated in the brain during epileptogenesis, resulting in the establishment of a persistent inflammatory condition that contributes to the generation of seizures. Furthermore, an unmet clinical need in epilepsy research is the absence of biomarkers able to detect epileptogenesis in individuals at risk following an epileptogenic insult.

Therefore, the fundamental goals of the work described in this thesis were (1) to characterize resolution mechanisms versus neuroinflammation during epileptogenesis; (2) to evaluate the potential antiepileptogenic and disease-modifying effect of treatments aimed at boosting endogenous specialized mechanisms of resolution of inflammation and blocking specific pathways playing crucial roles in triggering and maintaining neuroinflammation during epileptogenesis; (3) to assess the potential role of HGMB1 as mechanistic biomarker of neuroinflammation, epileptogenesis and treatment response.

I used three different rodent models of epileptogenesis induced by SE. The analyses were performed at different time points post-SE representative of disease development: the acute phase of SE, preceding spontaneous seizure onset, the time of disease onset and the chronic epileptic phase. The analyses were performed in the hippocampus, main epileptogenic area in the three experimental models considered and in human TLE.

The work is divided in four main parts.

(1) To characterize the time of induction of the resolution response versus neuroinflammation during experimental epileptogenesis by RT-qPCR and immunohistochemistry and lipidomic analysis. Pro-resolving receptors were analyzed also in hippocampi from patients who died after SE or affected by pharmacoresistant epilepsy.

(2) To evaluate the therapeutic effects of anticipation and potentiation of endogenous resolution mechanisms using stable analogues of pro-resolving lipid and peptide

mediators. Treatments were performed during epileptogenesis and their effects were studied on neuroinflammation, cognitive deficits and spontaneous seizures.

(3) To assess the therapeutic effects of a combined anti-inflammatory treatment aimed at blocking the activation of relevant neuroinflammatory pathways during epileptogenesis. The effects of this treatment were studied on spontaneous seizure and neurodegeneration.

(4) To study the biomarker role of HMGB1 for epileptogenesis and for predicting the therapeutic response to anti-inflammatory drugs.

CHAPTER 3 – MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Adult male C57/BL6N mice (30 g), adult male Sprague-Dawley rats (250-300 g) or male Sprague–Dawley rats at postnatal day (PN) 21 (with PN1 defined as the day of birth) were housed at constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light-dark cycle and free access to food and water. Animals were purchased from Charles River (Calco, Italy). Adult animals were housed one per cage due to the presence of the EEG recording implant. Our laboratory experience showed that when mice or rats are housed two or more each cage may easily damage each other EEG implants thereby causing loss of EEG set up or damaging the connecting cables. To enrich the cage environment, nesting materials was added to each cage for mice or wooden sticks for rats (Hutchinson et al., 2005) during animal housing including the time of experimental procedures. Other materials (e.g. tunnel) are not compatible with the EEG recording devices required for EEG monitoring.

All experimental procedures were conducted in conformity with institutional guidelines that are in compliance with national (D.L. n.26, G.U. March 4, 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and were reviewed and approved by the intramural ethical committee.

3.2 ANIMAL CARE

Procedures involving animals were conducted at the IRCCS - Istituto di Ricerche Farmacologiche "Mario Negri" which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate - UNI EN ISO 9001:2008 - Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011

edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01).

3.3 JUSTIFICATION OF THE CHOICE OF *IN VIVO* MODELS OF EPILEPTOGENESIS

We used three rodent models of epileptogenesis induced by SE.

3.3.1 Intra-amygdala kainic acid in mice

This model of epileptogenesis has been extensively described and used (Diviney et al., 2015; Iori et al., 2017b; Jimenez-Mateos et al., 2012; Mouri et al., 2008). Epilepsy develops in 100% adult C57/BL6N mice exposed to at least 1.5 h SE induced by intra-amygdala injection of kainic acid. This chemoconvulsant is a potent glutamate analog, acting as an agonist of the ionotropic glutamate AMPA/Kainate receptor subtype. The local and systemic injection of kainic acid induces seizures associated with neuronal cell death in forebrain, particularly in pyramidal cells and the hilus of dentate gyrus (Ben-Ari and Cossart, 2000).

Spontaneous motor generalized seizures develop after a short latency phase after SE, and their daily number is relatively stable for the first 60 days after epilepsy onset, then seizure frequency increases (in the 80% of mice) by about 3-fold after 2 months, reaching a stable baseline thereafter (Iori et al., 2017b). Mice developed cognitive deficits in the T-maze, and anxiety-like behaviors (Liu et al., 2013). Progressive neuronal cell loss occurs in forebrain mostly unilaterally to the injected amygdala (Mouri et al., 2008). This model will be used: (a) to characterize resolution versus neuroinflammation during epileptogenesis, and (b) to study effects of drugs administered after SE onset on neuroinflammation, cognitive deficits and spontaneous seizures (data reported in Chapter 4 and 5).

3.3.2 Electrical *status epilepticus* (SE) in adult rats

This model of epileptogenesis has been extensively described and used (Bertram and Cornett, 1993, 1994; De Simoni et al., 2000; Lothman et al., 1989; Noe et al., 2013; Pauletti et al., 2017; Vezzani et al., 1999b). Epilepsy develops in 100% adult Sprague Dawley rats exposed to SE induced by electrical stimulation of the CA3 region of the ventral hippocampus.

The advantage of using electrical stimulation model of SE is the possibility to stop the stimulation as soon as SE becomes self-sustained, i.e. when SE continues after the epileptogenic stimulus is withdrawn. This feature allows tight control of the severity of SE, thus reducing the mortality (~10%), giving a high percentage of animals (>90%) that develop self-sustained SE and become chronically epileptic. Moreover, this model is suitable for experimental studies during the prodromal phase before disease onset since it is quite long and, as previously reported, the 100% of rats develops epilepsy.

After 14.3 ± 3.1 days on average (n=6) during which no EEG or behavioural seizures occur, rats develop recurrent, generalized spontaneous seizures as demonstrated by video-EEG recording (Noe et al., 2013). Moreover around 80% of rats show 5-fold increase on average in the frequency of spontaneous recurrent seizures during 4.5 months, denoting disease progression (data reported in Chapter 6). Progressive neuronal cell loss occurs in forebrain (Noe et al., 2013). This model will be used: (a) to study disease-modification effects of drugs administered starting 1 h after SE onset for 1 week in order to cover the early phase of epileptogenesis before disease onset, and (b) to characterize modifications of HMGB1 and its isoforms during disease development (data reported in Chapter 6 and 7).

3.3.3 Pilocarpine-induced SE in PN21 rats

This model of epileptogenesis has been extensively described and used (Dube et al., 2001; Leroy et al., 2011; Marcon et al., 2009; Pascente et al., 2016; Roch et al., 2002). In this model, SE is induced by injection of lithium+pilocarpine in PN21 rats. Pilocarpine is a chemoconvulsant

that activates muscarinic acethylcoline receptors, increasing neuronal excitability, thus inducing SE. PN21 rats are used since it has been shown that the development of epilepsy depends on the age at which SE is induced in the rat (Scantlebury et al., 2007). In particular, epileptogenesis develops if SE is induced in at least PN12 rats (Kubova et al., 2004; Scantlebury et al., 2007). Most importantly, only a fraction of rats (~60-70%) exposed to SE at PN21 develops seizures in the adulthood (Marcon et al., 2009; Roch et al., 2002).

Previous data showed that the onset of SE after the administration of pilocarpine is around 16.0 ± 1.1 min, its duration is 22.6 ± 0.5 h, the temporal distribution of spikes and the power spectral densities during the first 3 h after SE onset were similar in all injected animals (n=8) (Pascente et al., 2016). Spontaneous seizures develop at around 70 days after SE onset (68.8 ± 1.8 days, n=5) (Dube et al., 2001; Leroy et al., 2011; Pascente et al., 2016). At 7 months post-SE rats with (*epileptic*) and without (*non-epileptic*) spontaneous seizures are identified by 2 weeks-EEG recording and by the hippocampal afterdischarge threshold (ADT) as a measure of focal excitability. Between 60-70% of rats (20 out of 31 rats) develops spontaneous seizures (5.8 ± 1.5 seizures over 2 weeks) (Dube et al., 2001; Leroy et al., 2011; Pascente et al., 2016). This model is highly valuable for determining the factors associated with epileptogenesis that may differentiate rats that eventually develop or do not develop spontaneous seizures. Thus, this model will be used to study HMGB1 as a biomarker of epileptogenesis (data reported in Chapter 7).

In these three *in vivo* models histopathological changes in the hippocampus are similar to those observed in human mesial TLE including neuronal loss, granule cell dispersion and mossy fiber sprouting (Bouilleret et al., 1999; De Simoni et al., 2000; Kralic et al., 2005; Noe et al., 2013; Pascente et al., 2016; Suzuki et al., 2005).

3.4 *IN VIVO* MODELS OF EPILEPTOGENESIS

3.4.1 Intra-amygdala kainic acid in mice

Mice were implanted under 1.5% isoflurane anaesthesia with a 23-gauge cannula unilaterally positioned on top of the dura mater for the intra-amygdala injection of kainic acid (Figure 3.1A; from bregma, mm: nose bar 0; AP -1.0, L -2.8; Franklin and Paxinos, 2008). A bipolar Teflon-insulated stainless-steel depth electrode (60 µm OD) was implanted in the dorsal hippocampus ipsilateral to the injected amygdala (from bregma, mm: nose bar 0; AP –1.8; L -1.5; -2.0 below dura mater; Franklin and Paxinos, 2008). Additionally, a cortical electrode was implanted onto the somatosensory cortex in the contralateral hemisphere. One or two additional guide cannulae were positioned on top of the dura mater (from bregma, mm: nose bar 0; AP 0, L -0.9; Franklin and Paxinos, 2008) ipsilateral (if only one) to the injected amygdala for intracerebroventricular (icv) injections of synthetic analogues of pro-resolving mediators or their vehicles. Finally, two screw electrodes were positioned over the nasal sinus and the cerebellum, and used as ground and reference electrodes, respectively (Figure 3.1B). Electrodes were connected to a multipin socket and secured to the skull by acrylic dental cement (Figure 3.2B).

One week after surgery, mice were connected to the EEG set up the day before SE induction in order to have a baseline EEG recording (Figure 3.1C). Kainic acid (0.3 µg in 0.2 µl; Sigma-Aldrich, Saint Louis, MO, USA, #K0250) was dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and injected in the right basolateral amygdala in freely moving mice using a needle protruding of 4.1 mm below the implanted cannula (Figure 3.1A). SE developed after approximately 10 min from kainic acid injection, and was defined by the appearance of continuous spikes with a frequency >1.0 Hz. After 40 min from SE onset, mice received diazepam (10 mg/kg, ip) to improve their survival. After SE induction mice were recorded at least for 24 h in order to evaluate SE duration (Figure 3.1C).

One group of mice was continuously (24/7) video-EEG recorded from the onset of SE until the onset of epilepsy (disease onset, Figure 3.1C) and for two additional weeks in order to evaluate the effect of the pro-resolving mediator on spontaneous seizures (data in Chapter 5) (Figure 3.1C). Mice were sacrified at different time points after SE for biochemical or immunohistochemical analysis depending on the experimental protocol (details regarding experimental groups in Chapter 4 and 5).

Mice implanted with electrodes and guide cannulae were used as SHAM control. They were vehicle-injected and not exposed to SE. They were used for evaluation of cognitive deficits and brain histological or biochemical analysis.

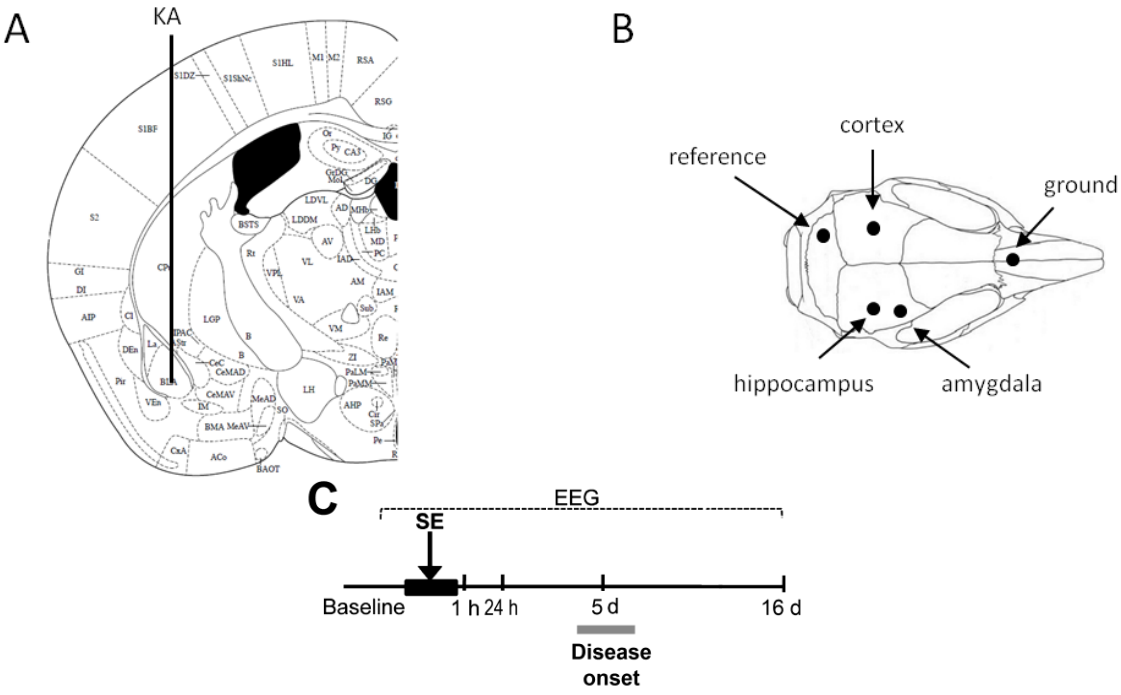


Figure 3.1. Intra-amygdala kainic acid mouse model of epileptogenesis. *Panel (A): kainic acid injection site is depicted by the arrow in the right basolateral amygdala. Panel (B): a schematic representation of the localization of recording electrodes and cannulae in mice brain. Three screw electrodes were used respectively as reference, ground and cortical recordings. One deep electrode was used for intra-hippocampal recording. One cannula was placed above the amygdala for kainic acid injection. One/two cannulae (not shown) were implanted above lateral ventricles for drug injections. Panel (C): schematic representation of disease development. Mice were EEG recorded for 24 h before SE induction (Baseline) and 24 h thereafter (Chapter 4 and 5). One group of mice was continuously (24/7) EEG recorded from the onset of SE until the onset of epilepsy (disease onset) followed by two additional weeks in order to evaluate the effect of the pro-resolving mediator on spontaneous seizures (Chapter 5).*

3.4.2 Electrical SE in adult rats

Rats were implanted under 1.5% isoflurane anaesthesia with two bipolar Teflon TM-insulated stainless-steel depth electrodes placed bilaterally into the temporal pole of the hippocampus (from bregma, mm: AP - 4.7; L - 5.0; - 5.0 below dura; Paxinos and Watson, 2005). Two screw electrodes were positioned over the nasal sinus and the cerebellum, and used as ground and reference electrodes, respectively. Electrodes were connected to a multipin socket and secured to the skull by acrylic dental cement (Figure 3.2A,B). Rats were allowed to recover from surgery in their home cage for one week. Before electrical stimulation, EEG baseline hippocampal activity was recorded in freely-moving rats for 24 h. Then, rats were unilaterally stimulated (50 Hz, 400 μA peak-to-peak, 1 ms biphasic square waves in 10 s trains delivered every 11 s) in the CA3 region of the ventral hippocampus for 60–90 min to induce SE according to a well-established protocol (De Simoni et al., 2000; Noe et al., 2013). EEG was recorded in each rat every 10 min epoch for 1 min in the absence of electrical stimulation, i.e. the 'stimulus-off' period. All rats used for subsequent analysis showed an EEG pattern of uninterrupted bilateral spikes in the hippocampi during the 'stimulus-off' period, starting between the first and the fourth epoch of stimulation onwards. These criteria selected rats developing SE that remitted spontaneously within 24 h from the initial stimulation then leading to subsequent epilepsy development (Bertram and Cornett, 1994; De Simoni et al., 2000; Lothman et al., 1989; Noe et al., 2013).

Rats were video-EEG recorded continuously (24/7) from the onset of SE until the onset of epilepsy (*prodromal phase,* Figure 3.2C). After the onset of spontaneous seizures (*disease onset,* Figure 3.2C), rats were temporarily disconnected by the EEG set up, and left in their home cage. EEG monitoring was resumed at 2 and 4 months post-SE (*chronic phase,* Figure 3.2C) to determine spontaneous seizure frequency by continuous video-EEG monitoring for 2 weeks (24/7). At the end of video-EEG recording, rats were sacrified for biochemical analysis of HMGB1 levels and immunohistochemical analysis of cell loss (details regarding experimental

groups in Chapter 6). Additional rats were sacrified at different time points following SE for biochemical or immunohistochemical analysis of HMGB1 levels and expression (details regarding experimental groups in Chapter 7).

Rats implanted with electrodes were used as SHAM controls. They were injected with vehicles and not electrically stimulated. They were used for blood HMGB1 measurements and histological brain analysis.

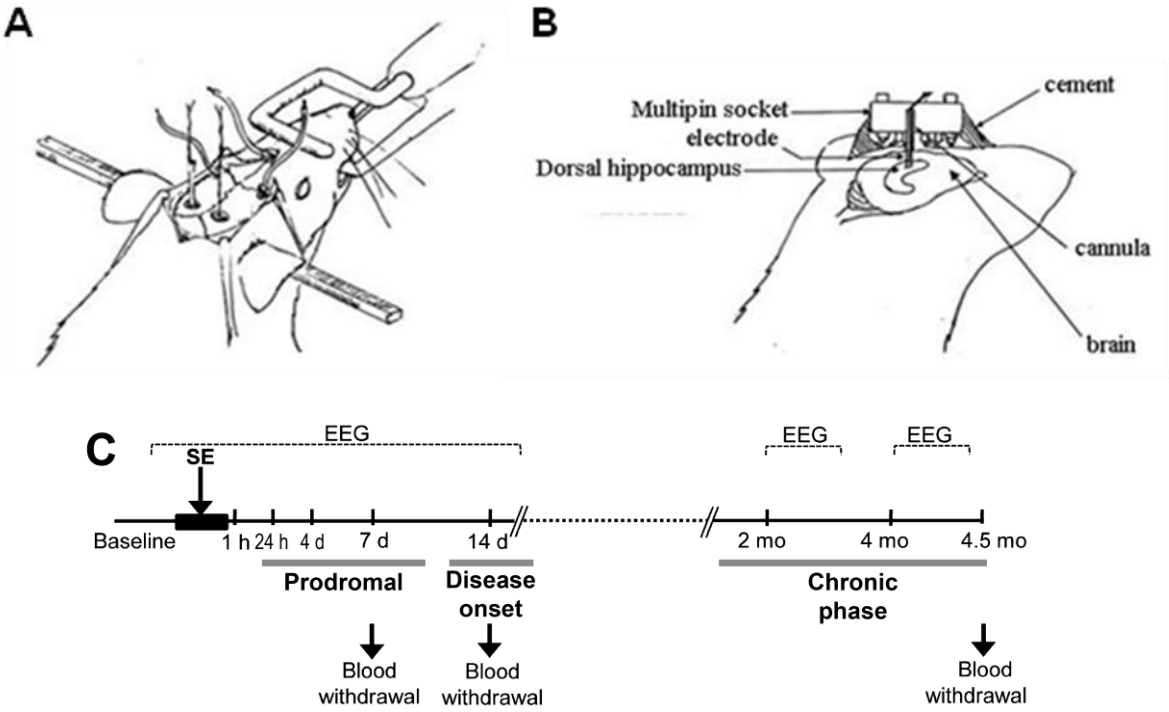


Figure 3.2. Rat model of epileptogenesis induced by SE evoked by electrical stimulation of ventral hippocampus. *Panel (A): schematic representation of the cannula and recording electrodes implant in rats using stereotaxic guidance. Panel (B): representation of the multipin socket secured to the skull by dental cement. Panel (C): schematic representation of disease development. Time of blood withdrawal for HMGB1 measurement is reported (details in Chapter 6 and 7).*

3.4.3 Pilocarpine-induced SE in PN21 rats

Lithium chloride (3 meq/kg; Merck Sharp & Dohme, Rome, Italy, #105679) was intraperitoneally injected in PN20 rats, 18 h before the subcutaneous injection of pilocarpine (60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA, #P6503) (Marcon et al., 2009; Roch et al., 2002). Controls were age-matched rats injected with lithium chloride and with an equivalent volume of vehicle (PBS, pH 7.4) instead of pilocarpine. All animals received an injection of 10% sucrose in PBS, 2 h after the onset of SE to improve hydration. SE was not interrupted by any drug administration. As in both adult and pediatric populations (DeLorenzo et al., 1995; Logroscino et al., 1997; Raspall-Chaure et al., 2006), the mortality rate in this SE model is relatively high (35%) (Pascente et al., 2016). SE was visually observed and its onset time and severity were measured in each rat for 6 h after pilocarpine injection. The following score was used in each animal: score 1 (general automatisms and exploratory behavior) and score 2 (stage 5 seizures, bilateral forelimb clonus with rearing and falling). For each score, was measured the time of appearance of the first symptoms. The onset of SE was determined by time of appearance of at least two consecutive stage 5 seizures (<5 min apart) that subsequently recurred for the duration of the observation period. Behavioral SE was characterized by continuous bilateral forelimb clonus with rearing and falling (stages 5 of Racine's scale). Spontaneous seizures develop after around 70 days from SE onset (*disease onset,* Figure 3.3). At day 75 post-SE these rats were electrode-implanted under 1.5% isoflurane anesthesia with a depth electrode in the septal pole of the hippocampus (from bregma, mm: AP –3.3; L -2.4; –3.0 below dura; Paxinos and Watson, 2005) and a contralateral surface cortical screw electrode. Two screw electrodes were positioned over the nasal sinus and the cerebellum, and used as ground and reference electrodes, respectively. Electrodes were connected to a multipin socket and secured to the skull by acrylic dental cement (Figure 3.2B). Rats were video-EEG monitored at 7 months post-SE for 2 weeks (24/7) for identifying animals developing or not developing epilepsy (representative time point of the *chronic phase,* Figure 3.3). At the end of monitoring, rats were tested for their

hippocampal threshold to electrically induced ADT. Animals were electrically stimulated via the recording hippocampal electrode, using constant current stimulus (1 msec monopolar square waves, 50 Hz for 2 s). Determination of the ADT was done using an ascending stepwise procedure (Frigerio et al., 2012; Pascente et al., 2016). The initial stimulus intensity was 50 μA and was increased by 10 μA up to 100 μA, then by 100 μA up to a maximal value of 400 μA by intervals of 1 min until one AD of at least 10 s duration was elicited. Twenty-four hours after AD induction, rats were sacrified for immunohistochemical analysis of HMGB1 and cell loss. Additional rats were sacrified at different time points following SE for biochemical analysis of blood HMGB1 levels (for details regarding experimental groups, see Chapter 7).

SHAM animals were vehicle-injected (not exposed to SE) and used as controls for blood HMGB1 measurements and histological brain analysis.

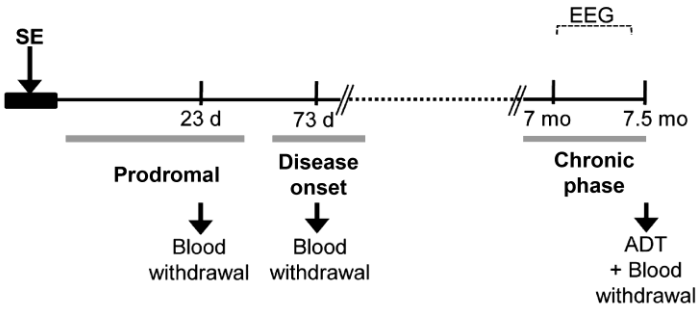


Figure 3.3. Schematic representation of disease development in pilocarpine-induced SE model in PN21 rats. *The prodromal phase includes the period between SE and the onset of spontaneous seizues (disease onset). Time of blood withdrawal for HMGB1 measurement is reported (details in Chapter 7).*

3.4.4 SE and spontaneous recurrent seizure assessment and quantification

SE was defined by the appearance of continuous spike activity with a frequency >1 Hz intermixed with high amplitude and frequency discharges lasting for at least 5 s, with a frequency of >8 Hz. Spikes were defined as sharp waves with amplitude at least 2.5-fold higher than the standard deviation of baseline signal and duration lower than 100 ms, or as a spike-and-wave with duration lower than 200 ms (Pitkanen et al., 2005). The end of SE was defined by the occurrence of interspike intervals longer than 1 s. SE was evaluated by EEG analysis and its total duration and the number of spikes were quantified during the first 24 h using Clampfit 9.0 program (Axon Instruments).

Epilepsy onset is defined with two unprovoked spontaneous seizures occurring at least 48 h from the end of SE. Spontaneous seizures are EEG paroxysmal events always accompanied by generalized motor convulsions. We reckoned the total number of spontaneous seizures during each 2-weeks recording period, and divided them by 15 days to estimate the number of daily seizures. EEG activity was recorded using the Twin EEG Recording System connected with a Comet AS-40 32/8 Amplifier (sampling rate 400 Hz, high-pass filter 0.3 Hz, low-pass filter 70 Hz, sensitivity 2000 mV/cm; Grass-Telefactor, West Warwick, R.I., USA). Digitized EEG data were processed using the Twin record and review software.

3.5 NOVEL OBJECT RECOGNITION TEST (NORT)

This test was used to study the effects of $PD1_{n\text{-}3DPA}$ on the encoding, consolidation and retrieval of long-term non-spatial object memory, as described earlier (Balducci et al., 2010). The test was performed in the open-square gray arena (40×40 cm) surrounded by 30-cm high wall, with the floor divided into 25 equal squares by black lines. Mouse behavior was remotely monitored via video camera. All experiments were started between 9:00 and 10:00 am. Twenty-four hours *prior* to the test, mice were allowed to habituate in the arena for 5 min (habituation phase;

Figure 3.4). The test began on the next day with the familiarization phase, when mice were placed into the open field for 10 min in the presence of two identical objects positioned in internal nonadjacent squares. The following objects were randomly used: black plastic cylinders (4×5 cm); transparent scintillation vials with white cups (3×6 cm); metal cubes (3×5 cm). Cumulative exploration time of both objects and of each object separately was recorded. Exploration was defined as sniffing, touching, and stretching the head toward the object at a distance of not more than 2 cm. Twenty four hours after familiarization, the recognition phase of the test was performed: mice were placed for 10 min in the open field which contained one object presented during the familiarization phase (familiar object) and a novel unfamiliar object. The time spent exploring novel (N) and familiar (F) objects, as well as cumulative exploration time (i.e. novel+familiar, N+F) was recorded. Novel object recognition was quantified using the discrimination index (N-F/N+F): the difference between the time spent exploring the novel and the familiar objects (N-F) divided by the sum of total exploration time (N+F) (Okun et al., 2010).

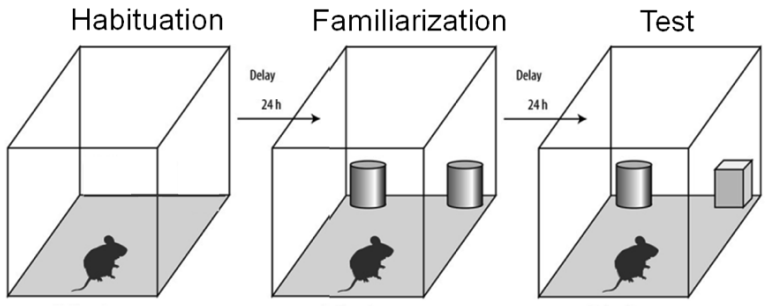


Figure 3.4. Novel object recognition test (NORT). *During habituation mice explored an empty arena for 5 min. During the familiarization session (24 h after habituation), mice freely explored two identical objects for 10 min. After 24 h, a novel object and a familiar one were used for the test session (10 min, test).*

3.6 IMMUNOHISTOCHEMISTRY

3.6.1 Immunohistochemical studies from *in vivo* preparations

Mice and rats were deeply anaesthetized using ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) and perfused via ascending aorta with 50 mM cold PBS, pH 7.4 followed by chilled 4% paraformaldehyde (Merck, Darmstadt, Germany, #104005) in PBS. The brains were post-fixed for 90 min at 4°C, and then transferred to 20% sucrose in PBS for 24 h at 4°C. The brains were immersed in -45°C isopentane for 3 min and stored at -80°C until assayed.

Serial coronal sections were cut on a cryostat throughout the septo-temporal extension of the **mouse** hippocampus (-0.94 to -3.64 mm from bregma; Franklin and Paxinos, 2008). I prepared 4 series of 16 sections each and in each serie we stained the slices as follows: the 1st slice for IL-1β, the 2nd for TNF-α, the 3rd for Annexin A1, the 4th for ALXR and the 5th for ChemR23 (data reported in Chapter 4). Three slices per mice were used for each marker.

Serial horizontal (electrical-SE model) or coronal (pilocarpine-induced SE model) sections (40 µm) were cut on a cryostat throughout the temporal or the septo-temporal extension of the **rat** hippocampus (-4.6 to -7.6 mm or -2.0 to -6.0 mm from bregma; Paxinos and Watson, 2005). I prepared 7 series of 10 sections each. In each serie, the 1st and 5th sections were stained for Nissl to assess neuronal cell loss. In the 2nd and 5th series, the 2th section was stained for HMGB1 (data reported in Chapter 6 and 7). Four slices per rat were used for each marker.

The same anatomical structures were retained within each series of sections.

3.6.1.1 IL-1β, TNF-α and HMGB1

Slices were incubated at 4°C for 10 min in 70% methanol and 2% H_2O_2 in Tris-HCl-buffered saline (TBS, pH 7.4), followed by 30 min incubation in 10% fetal bovine serum (FBS), 1-10% bovine serum albumin (BSA) in 1% Triton X-100 in TBS. Then, the slices were incubated overnight with the primary antibody against **IL-1β** (1:200, Santa Cruz Bio., CA, USA, #sc-1252) or **TNF-α** (1:1000, Peprotech, NJ, USA, #500-P64G) at 4°C in 10% FBS, 1-10% BSA in 1% Triton X-100 in TBS. **HMGB1**

immunostaining was carried out as previously described (Ravizza and Vezzani, 2006). Briefly, sections were incubated at 4°C for 60 min in 10% normal goat serum (NGS) in 0.1% Triton X-100 in PBS. Then the slices were incubated with the primary antibody against HMGB1 (1:1000, Abcam, Cambridge, UK, #AB18256) overnight at 4°C in 4% NGS in 0.1% Triton X-100 in PBS. Immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vector Labs, USA). The sections were reacted using 0.4 mM 3,3-diaminobenzidine (DAB; Sigma, #D8001) in 50mM Tris-HCl–buffered saline, pH 7.4, and 0.01% H_2O_2, and the signal was amplified using nickel ammonium. After DAB incubation, three 5-min washes were done with TBS, then sections were mounted onto gelatin-coated slides and dried overnight at room temperature. They were dehydrated and coverslipped the next day. No immunostaining was observed by incubating the slices with the primary antibodies preabsorbed with the corresponding peptides (1 µM, 24 h at 4°C), or without the primary antibodies (Ravizza and Vezzani, 2006; Vezzani et al., 1999a). The primary antibodies and experimental procedures have been previously demonstrated to determine a specific immunohistochemical signal of the protein of interest (IL-1β, TNF-α and HMGB1) in rodent brain slices (Ravizza and Vezzani, 2006; Vezzani et al., 1999a).

3.6.1.2 ALXR, ChemR23 and Annexin A1

Free-floating sections were rinsed for 15 min in FBS 3%, BSA 10% in 100 mM PBS at 4°C, followed by overnight incubation with the primary antibody anti-**ALXR** (1:3000, Novus Biologicals, CO, USA, #NLS1878) or anti-**ChemR23** (1:200, Santa Cruz, CA, USA, #SC-32652) at 4°C in 3% FBS and 10% BSA diluted in PBS.

For **Annexin A1** immunohistochemistry, antigen retrieval was performed using a microwave in Tris-EDTA buffer (10 mM Tris + 1 mM EDTA, pH 9) at 160 Watt for 5 min. Then, free-floating sections were rinsed for 10 min in 1% H_2O_2 in PBS pH 7.4, followed by 3 washes of 5 min each in PBS pH 7.4. Following a 60 min incubation in 10% NGS, 3% BSA in 0.1% Triton X-100 in PBS at 4°C,

the slices were incubated overnight at 4°C with the primary antibody against Annexin A1 (1:500, Thermo Scientific, MA, USA, #PA5-27315).

After three 5-min washes in PBS, immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, #PK6100). Immunoreactivity was tested as described previously for the other proteins (Section 3.6.1.1). No immunostaining was observed by incubating the slices with the primary antibody preabsorbed with the corresponding peptide at concentrations exceeding 10- to 300-fold antibody concentration (24 h at 4°C) or without the primary antibody (negative control).

Quantitative analysis of ALXR and ChemR23 staining was done by counting the total number of positive neuronal and astrocytic cells in DAB coloured sections. The quantification was performed in 3 slices per each mouse in one field representing the CA1 area captured at 20X magnification. ALXR- or ChemR23-positive cells were marked and an automated cell count was generated using Fiji software. Data obtained in each section/area/mouse were averaged, thus providing a single value per mouse, and this value was used for the statistical analysis.

3.6.1.3 Double and triple immunostaining

Three additional **mice** were perfused at 72 h after SE for double or triple immunostaining to identify the cells expressing IL-1β, TNF-α, ALXR, ChemR23 and Annexin A1 (data in Chapter 4). One brain slice was used for each cell type marker. After incubation with the primary antibodies, slices were incubated in biotinylated secondary anti-goat (for IL-1β, TNF-α and ChemR23) or anti-rabbit antibodies (ALXR and Annexin A1) (1:200, Vector Labs), then in streptavidin–horseradish peroxidase (HRP) and the signal was revealed with tyramide conjugated to Fluorescein using TSA amplification kit (NEN Life Science Products, Boston, MA, USA). Sections were subsequently incubated with the following primary antibodies: mouse anti-GFAP (1:2500, Chemicon, Temecula, CA, USA, #MAB3402), a marker of astrocytes, or rat anti-CD11b (1:1000, MAC-1, Serotec; #MCA719, Clone 5C6), a marker of microglia, or mouse anti-

NeuN (1:1000, Chemicon, #MAB377), a marker of neurons, or rabbit anti-NPY (1:25000, Peninsula Lab., CA, USA, #T-4070) to identify interneurons. Moreover, I performed a double-staining analysis of IL-1β with ALXR to study if their expression co-localized in the same cell population.

Two **rats** for each time point were randomly chosen for triple immunostaining to identify the cells expressing HMGB1 (data in Chapter 6). Two brain slices adjacent to those used for HMGB1 immunohistochemistry were used for each cell type marker. After incubation with the primary HMGB1 antibody, slices were incubated with an anti-rabbit biotinylated secondary antibody (BA-1000; 1:200, Vector Labs, Burlingame, CA, USA), then in streptavidin–HRP and the signal was revealed with tyramide conjugated to Fluorescein using TSA amplification kit (NEN Life Science Products, Boston, MA, USA, #SAT-701001EA). Sections were subsequently incubated with the following primary antibodies: mouse anti-GFAP (1:3500, Merck Millipore, Livingston, UK, #MAB3402), or mouse anti-OX-42 (1:1000, Serotec, Oxford, UK, #MCA275G), a marker of microglia, or with mouse anti-rat endothelial protein (EBA, 1:10000, Sternberger, Lutherville, MD, USA, #SMI-71R) to identify microvessels.

Fluorescence was detected using anti-mouse or anti-rat secondary antibody conjugated with Alexa546 (1:250; Molecular Probes, Leiden, The Netherlands, #A-11030). Additional incubation in Hoechst 33258 (1:500; Molecular Probes, #H3569) in PBS for 10 min was done to visualize the cell nucleus.

Slide-mounted sections were examined with an Olympus Fluoview laser scanning confocal microscope (microscope BX61 and confocal system FV500) using excitations of 488 nm (Ar laser), 546 nm (He-Ne green laser) and 350 nm (ultraviolet) for fluorescein, Alexa546 and Hoechst, respectively. The emission of fluorescent probes was collected on separate detectors. To eliminate the possibility of bleed-through between channels, the sections were scanned in a sequential mode.

3.6.2 Quantification of neuronal cell loss

Cell loss was measured in Nissl stained slices from rat sacrified at the end of experiments (data reported in Chapter 6 and 7), as previously described (Noe et al., 2013; Pascente et al., 2016). Briefly, images of the temporal (electrical-SE model) or the septo-temporal (pilocarpine-induced SE model) pole of the hippocampus in each hemisphere were captured at 20X magnification using a BX61 microscope equipped with motorized platform (Virtual Slider Microscope, Olympus, Germany) and digitized. Quantification was done in 4 Nissl-stained horizontal or coronal sections for each rat brain/emisphere. Neuronal cell loss in the hippocampus was quantified by reckoning the number of Nissl-stained neurons in CA1 and CA3 pyramidal cell layers and the hilar interneurons. The number of neurons in entorhinal and frontoparietal cortices, was reckoned only in the temporal pole slices (electrical-SE model) in two different fields covering cortical layer III in each hemisphere (274 x 165 µm for entorhinal cortex and 414 x 176 µm for frontoparietal cortex) captured at 20X and 10X magnification, respectively. Nissl-positive cells were marked and an automated cell count was generated using Fiji software. Data obtained in each section/area/rat were averaged, thus providing a single value for each area per rat, and this value was used for the statistical analysis.

3.6.3 ALXR and ChemR23 immuhistochemistry in human tissue

Human brain tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin embedded tissue was sectioned at 5 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel, Braunschweig, Germany) and processed for immunohistochemical staining. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 95%, 70%) and incubated for 20 min in 0.3% hydrogen peroxide diluted in methanol. Antigen retrieval was performed using a pressure cooker in Tris-EDTA buffer (10 mM Tris + 1mM EDTA, pH 9) at 120°C for 10 min. Slides were washed with 0.1 M PBS (pH 7.4) and incubated overnight with primary antibody anti-ALXR (1:500, Novus Biologicals, CO, USA, #NLS1878) and anti-ChemR23 (1:50, Santa Cruz, CA, USA,

#SC-32652) in PBS at 4°C. After washing with PBS, sections were stained with a polymer based peroxidase immunohistochemistry detection kit (Brightvision plus kit, ImmunoLogic, Duiven, The Netherlands) according to the manufacturer's instructions. Staining was performed using Bright DAB substrate solution (1:10 in 0.05 M Tris-HCl, pH 7.6; ImmunoLogic, #BS04-999) with 0.015% H_2O_2. Sections were dehydrated in alcohol and xylene and coverslipped.

Semi-quantitative analysis of staining was done by calculating the immunoreactivity score as the product of intensity of staining x frequency of stained cells. Intensity: 0=negative, 1=weak, 2=moderate, 3=strong. Frequency: 1=single to 10%, 2=11-50%, 3=>50%.

Immunohistochemistry analysis on human tissue was performed by Dr. Erwin Van Vliet, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

3.7 BIOCHEMICAL ANALYSIS

3.7.1 RNA isolation and real-time quantitative polymerase chain reaction analysis

Mice (see Chapter 4 and 5) were injected with kainic acid (Section 3.4.1), underwent SE and were sacrified at different time points post-SE onset. Mice were deeply anesthetized using ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), then perfused via ascending aorta with 50 mM ice-cold PBS (pH 7.4) for 1 min to remove blood, and decapitated. The hippocampus ipsilateral to the injected amygdala was rapidly dissected out at 4°C in a RNAse free environment, immediately frozen in liquid nitrogen and stored at –80°C until assay. For RNA isolation, frozen hippocampi were homogenized in Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands, #79306). The total RNA including the miRNA fraction was isolated using the miRNeasy Mini kit (Qiagen Benelux, Venlo, the Netherlands, #217004) according to manufacturer's instructions. The concentration and purity of RNA were determined at 260/280 nm using a high-speed microfluidic UV/VIS spectrophotometer QIAxpert (Qiagen, Milano, Italy).

Real-time quantitative polymerase chain reaction analysis (RT-qPCR) using Taqman or SYBR Green assays was performed in Chapter 4 and Chapter 5, respectively.

cDNA was synthesized from 500 ng RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, California, USA, #4368814) in a total volume of 20 µl following the manufacturer's protocol (Applied Biosystems, California, USA).

In Chapter 4, Taqman RT-qPCR was performed with the CFX PCR System from Biorad. cDNA was analysed in triplicate using Applied Biosystems TaqMan gene expression assays. Briefly, qPCR reactions were performed in a 384 well plate using 5 µl Universal Master Mix (Life Technologies #43004437), 2.5 µl of DEPC treated water, 0.5 µl of gene expression assay and 2 µl cDNA per well. Cq values were obtained using automatic threshold and baseline and analysed using qBase+ software. Data were normalized using geometric mean of 3 independent house-keeping genes (mfsd5, brap, bcl2l13).

In Chapter 5, cDNA was analysed using QuantiFast SYBR Green PCR Master Mix (Qiagen, Milano, Italy, #204054). Samples were run in triplicate to assess technical variability using 384-well reaction plates in an ABI PRISM 7900 HT (Applied Biosystems). Data were normalized using geometric mean of 3 independent house-keeping genes (hprt1, sv2b, pdk1).

RT-qPCR measurements by Taqman assays were performed by Catherine Vandenplas, Molecular Biology and Gene Expression, UCB BioPharma, Braine l'Alleud, Belgium. Measurements by SYBR Green assays were performed by Ilaria Craparotta, Translational Genomic Unit, Department of Oncology, IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy.

3.7.2 Assessment of LXA_4 and RvD1 by enzyme-linked immunosorbent assay

Mice (n=24) were injected with kainic acid (Section 3.4.1), underwent SE and were sacrified 72 h post-SE onset (data in Chapter 4). Mice were deeply anesthetized and perfused as previously reported (Section 3.7.1). The hippocampus ipsilateral to the injected amygdala was rapidly dissected out at 4°C, immediately frozen on liquid nitrogen and stored at –80°C until assay. Total LXA_4 and RvD1 levels were determined by commercially available enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions (Oxford Biomedical Research, Oxford, MI, USA, #EA45; Cayman Chemicals, Ann Arbor, MI, USA, #500308). For LXA_4 measurements, 2 hippocampi ipsilateral to the injected amygdala were pooled together giving one sample/2 mice, while RvD1 was measured in one hippocampus/mouse. SHAM mice (n=16) were injected with vehicle instead of kainic acid in the basolateral nucleus of amygdala and 2 hippocampi from one mouse were pooled together for LXA_4 analysis, while one hippocampus/mouse was used for RvD1 analysis. For tissue extraction, hippocampal samples were homogenized in ethanol by a pestle homogenizer, followed by centrifugation at 1500 X *g* for 15 min. The supernatant was collected and acidified to pH 3.5. These samples were extracted through Sep-Pak C18 columns (Waters, #WAT051910). Briefly, the columns were equilibrated with methanol and ddH_2O, and acidified samples were loaded immediately. Bound lipids were eluted with methyl formate, brought to dryness by nitrogen gas, and finally resuspended by extraction buffer supplied with the ELISA kit. The color reaction was stopped by 1 M HCl and the absorbance was read at 450 nm (ELISA reader, Infinite M200, Tecan, Italy). The detection limit was <10-20 pg/ml.

3.7.3 Lipidomic analysis by liquid chromatography mass spectrometry

Mice (n=16) were injected in the basolateral nucleus of amygdala with kainic acid (Section 3.4.1), underwent SE and were sacrified 72 h post-SE onset (data in Chapter 5). Mice were

deeply anesthetized and perfused as previously reported (Section 3.7.1). The hippocampus ipsilateral to the injected amygdala was rapidly dissected out at 4°C, immediately frozen in liquid nitrogen and stored at –80°C until shipment. In order to have enough tissue for the analysis we pooled 2 hippocampi from 2 different mice, thus giving n=8 samples. SHAM mice (n=8) were injected with vehicle instead of kainic acid in the basolateral nucleus of amygdala and hippocampi from one mouse were pooled together.

Lipidomic analysis was performed by liquid chromatography mass spectrometry (LC/MS-MS) as previously described (Colas et al., 2014) in the laboratory of Dr. Jesmond Dalli, Lipid Mediator Unit, William Harvey Research Institute, London, UK.

3.7.4 Assessment of total HMGB1 by ELISA

Blood was collected as specifically reported in Chapter 7 and stored at –80°C until shipment. Non-identifiable blood samples were analyzed by an investigator blinded to the identity of the samples. Total HMGB1 level was determined by commercially available ELISA (Shino-test Corp, Sagamihara, Japan) according to the manufacturer's guidelines as described previously (Lehner et al., 2012). In brief, thawed samples were centrifuged at 2000xg for 1 min. The 96-well plate was coated with sample diluent to which 10 µl of sample was added in duplicate. Samples were incubated overnight at 37°C for 20 h. Plates were washed 5 times in wash buffer (400 µl/well) and air-dried. Detection antibody solution (100 µl/well) was added for 2 h at room temperature. Following the subsequent washing step, substrate solutions were added in equal parts (100 µl/well) and incubated at room temperature protected from light with foil seal for 30 min and the plate read at 450 nm. Results were fitted to the standard curve.

3.7.5 Analysis of HMGB1 isoforms by electrospray ionization LC/MS-MS

The ventral hippocampus ipsilateral to the electrical stimulation and blood collected from the same rats (details in Chapter 7) were used for LC/MS-MS analysis. Non-identifiable samples

were analyzed by an investigator blinded to the identity of the samples. All chemicals and solvents were of the highest available grade (Sigma Aldrich, Dorset, UK). Samples were precleared with 50 µl protein G-Sepharose beads for 1 h at 4°C. Supernatant HMGB1 was immunoprecipitated with 5 µg rabbit anti-HMGB1 (Abcam, Cambridge, UK, #ab18256) for 16 h at 4°C as previously described (Antoine et al., 2012). Free thiol groups within HMGB1 were alkylated for 90 min with 10 mM iodoacetamide at 4°C. Cysteine residues in disulfide bonds were then reduced with 30 mM DTT at 4°C for 1 h followed by alkylation of newly exposed thiol groups with 90 mM NEM at 4°C for 10 min. Samples were subjected to trypsin (Promega, Southampton, UK, #V5111) or GluC (New England Biolabs, Hitchin, UK, #P8100S) digestion according to manufacturer's instructions and desalted using ZipTip C18 pipette tips (Merck Millipore). Characterization of whole protein molecular weights, acetylated lysine residues, or redox modifications on cysteine residues within HMGB1 were determined as described previously by whole protein electrospray ionization or tandem mass spectrometry (Antoine et al., 2009, 2012) using either an AB Sciex QTRAP 5500 or an AB Sciex TripleTOF 5600 (Sciex Inc., Warrington, UK). Peptide analysis was determined using an AB Sciex QTRAP 5500 equipped with a NanoSpray II source by in-line liquid chromatography using a U3000 HPLC System (Dionex, Thermo Fisher UK Ltd., Hemel Hempstead, UK), connected to a 180 µm × 20 mm nanoAcquity UPLC C18 trap column and a 75 µm × 15 cm nanoAcquity UPLC BEH130 C18 column via reducing unions. A gradient from 0.05% TFA (v/v) to 50% ACN/0.08% TFA (v/v) in 40 min was applied at a flow rate of 200 nl/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 19, and the interface heater to 150°C. A schematic overview of the workflow of the analysis described in this paragraph is depicted in Figure 3.5.

Un-blinding and linkage to the clinical information was performed by a second investigator following completion of the analysis in all cases.

Analysis of total HMGB1 and its isoforms by ELISA and LC/MS-MS were performed by Dr. Daniel Antoine, Centre for Drug Safety Science, University of Liverpool, Liverpool, UK.

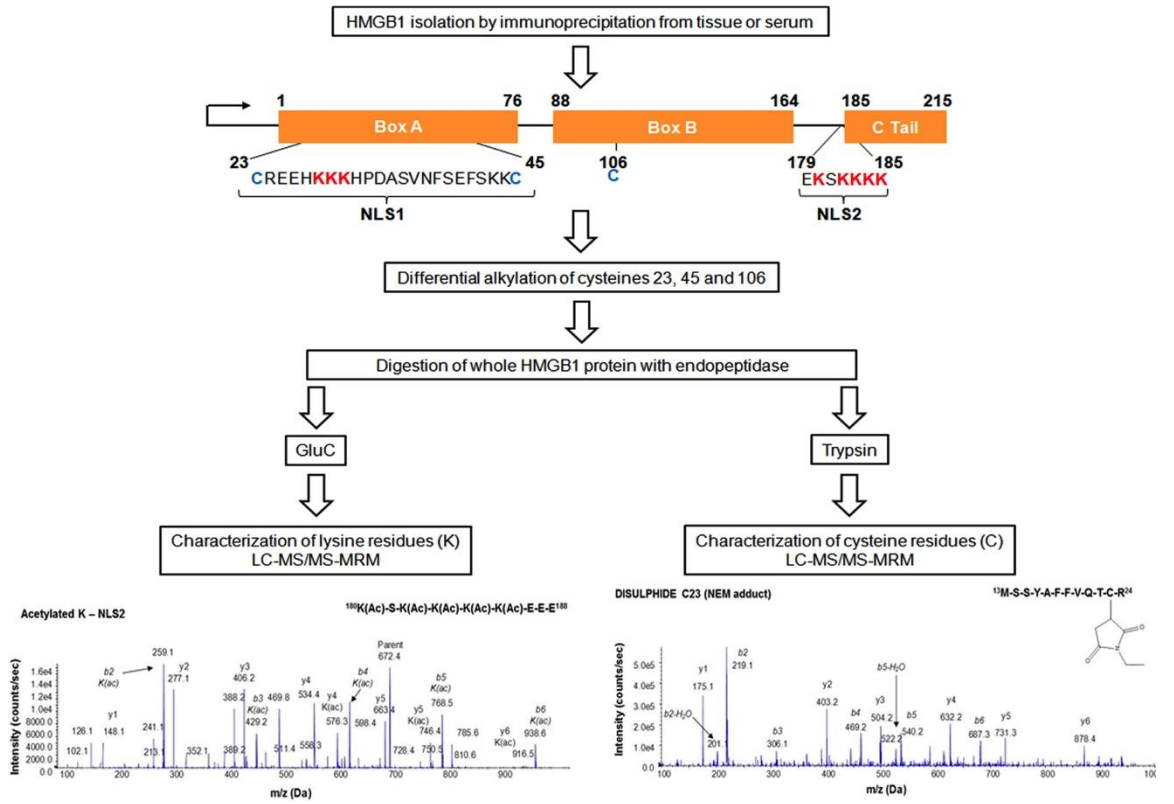


Figure 3.5. Schematic overview of the workflow to characterize and quantify post-translational modifications on HMGB1 isoforms *isolated from serum and brain tissue HMGB1 (representative molecular structure given with labelled amino acids with the one letter code) is isolated by immunoprecipitation and cysteine residues are alkylated by differential alkylation to lock in redox states as previously described* (Yang et al., 2012). *Half of the immunoprecipitation of HMGB1 is digested with endopeptidase trypsin. Resulting peptides are characterized by electrospray ionization LC/MS-MS and quantified by a Multiple Reaction Monitoring (MRM) protocol of an authentic heavy labelled peptide standard. For representation, a HMGB1 derived peptide following trypsin digestion spanning amino acids 13-24 containing cysteine 23 that has been alkylated with N-ethylmaleimide (NEM) is shown (1564.7 Da). Cysteines alkylated with iodoacetamide are characterized as fully reduced and those alkylated with NEM are characterized as participating in a disulfide bond* (Yang et al., 2012). *Since proteolytic cleavage by trypsin occurs following K residues, GluC digestion must be used to characterize K post translational acetyl-modifications in resulting peptides from the remaining fraction of the immunoprecipitation reaction. Therefore, C redox and K acetyl modifications cannot be achieved from the same digestion for HMGB1. A representative peptide sequence spanning K residues within Nuclear Localisation Sequence 2 (NLS2) derived following HMGB1 digestion with GluC is given (1342.6 Da). MS-MS analysis of b and y ions generated from these peptides confirms the amino-acid sequence of each peptide and also the addition of a specific post translational or alkylation modification. On each MS-MS spectra, b and y ions are labelled as required.*

3.8 STATISTICS

Quantification analysis in each experiment was done by two independent investigators blinded to the experimental groups.

In each animal experiment no *ad interim* analysis was done. Sample size was *a priori* determined based on previous experience with the respective models; endpoints (outcome measures) and statistical tests were prospectively selected. A simple random allocation using a web site randomization program (www.randomization.com) was applied to assign a subject to a particular experimental group. All efforts were made to minimize the number of animals used and their suffering according to the 3Rs use. Data acquisition and analysis was done blindly. Statistical analysis was performed by GraphPad Prism 6 (GraphPad Software, USA) for Windows using absolute values. Data are presented as mean ± s.e.m. (n=number of individual samples).

A parametric or non parametric test was used depending on the results of normality tests.

In Chapter 4, Mann Whitney U test for two independent groups in ELISA analysis of LXA_4 and RvD1 levels (Figure 4.3) and Kruskal-Wallis test with Dunn's post-hoc correction for more than two independent groups in RT-qPCR analysis (Figure 4.1-4.4) were used. Statistical analysis of semi-quantitative evaluation of immunohistochemical staining in human tissue was performed by Mann Whitney U test (Figure 4.5).

In Chapter 5, Kruskal-Wallis test with Dunn's post-hoc correction was used in RT-qPCR analysis (Figure 5.4, 5.7) and in weight measurements (Figure 5.3, 5.6). Based on normality test's results, t test was used for lipidomic analysis (Figure 5.5) and one way ANOVA with Tukey's post-hoc test for NORT data (Figure 5.8). One tailed t test was used for testing the possibility that the treatment with $PD1_{n\text{-}3DPA}ME$ was able to reduce spontaneous seizures (Figure 5.10), given the positive effects of this pro-resolving mediator on neuroinflammation and cognitive deficits.

In Chapter 6 and 7, non parametric test were used for statistical analysis of data: Mann–Whitney U test for two independent groups (Figure 6.2, 6.4) and Kruskal-Wallis followed by Dunn's post-hoc test for more than two independent groups (Figure 6.3, 6.5, 7.3, 7.4). The progression index (Figure 6.4) was calculated for each rat as the ratio between the total number of seizures experienced between 4.0 and 4.5 months divided by the total number of seizures from 2.0 to 2.5 months in the treatment over the vehicle-injected group. The odds (Figure 6.4) in each experimental group were calculated as the ratio between the number of seizures experienced by rats from 4.0 to 4.5 months and the total number of seizures from 2.0 to 2.5 months. The Odds Ratio was the ratio between the odds of treatment and vehicle groups.

Repeated measures one-way ANOVA was used to compare HMGB1 levels at different time points of disease development in the same group of rats (Figure 7.3, 7.5). The performance of HMGB1 as biomarker was assessed using non parametric Receiver Operating Characteristics (ROC) curves: the area under the curve (AUC) was calculated and compared with chance (AUC=0.5). The performance of the biomarker is considered excellent for AUC values close to 1, 0.8–0.9 is considered good, 0.7–0.8 is considered adequate, and <0.7 is considered poor (Table 7.1, 7.2).

Differences were considered significant with a $p<0.05$.

CHAPTER 4 - CHARACTERIZATION OF RESOLUTION MECHANISMS VS NEUROINFLAMMATION DURING EPILEPTOGENESIS

4.1 INTRODUCTION

Experimental and clinical observations suggest a lack of efficient endogenous anti-inflammatory responses in epileptogenic brain tissue. In particular, (a) IL-1β induction anticipates by several hours that of its receptor antagonist IL-1Ra (De Simoni et al., 2000); (b) inhibitory proteins of the complement system are induced to a lesser extent than those activating the same system (Aronica et al., 2007); (c) the expression of the transcription factor ATF3 that negatively regulates TLR4 expression is inversely related to the frequency of seizures while the opposite is observed for TLR4 (Pernhorst et al., 2013) which contributes to epileptogenesis (Maroso et al., 2010). This evidence suggests that a deficiency of anti-inflammatory mechanisms is a key contributor to the persistent, thus pathologic, neuroinflammation in epilepsy. Among the mechanisms apt to resolve the inflammatory response, there are specialized pro-resolving lipid and protein mediators that are able to drive an active resolution process. This phenomenon has been well described in peripheral inflammatory diseases while it is still scarcely explored in CNS disorders associated with a neuroinflammatory response, including epilepsy. Specialized pro-resolving lipids are biosynthetic products of LOX and are generated from fatty acids such as AA or n-3 polyunsaturated fatty acids, such as EPA and DHA. Pro-resolving lipids include LXs, Rvs and PDs. These lipids together with peptide ligands, like Annexin A1 and Chemerin, activate pro-resolving pathways by binding their specific G protein coupled receptors, such as ALXR and ChemR23, thus determining inhibition of MAPK and NF-KB activation. The final results of this response in the injured tissue are a reduced production of pro-inflammatory cytokines and recruitment of immune cells associated with increased synthesis of anti-inflammatory molecules. These mechanisms lead to the resolution of the inflammatory response and the re-establishment of tissue homeostasis (Serhan et al., 2008). If resolution is impaired or inefficient, the acute inflammatory response will persist and may become detrimental for the tissue.

In this study, we characterized the induction of resolution mechanisms vs neuroinflammation during epileptogenesis induced by SE. Firstly, we studied the mRNA and protein expression of key pro-inflammatory cytokines with a pivotal role in epileptogenesis mechanisms, namely, IL-1β and TNF-α. Secondly, we characterized the pro-resolving mechanisms by focusing on both peptide mediators (IL-1Ra and Annexin A1) and lipid mediators, such as LXA_4 and RvD1, and their biosynthetic lypoxigenase enzymes (LOX5 and 15). Finally, we investigated the expression of two main pro-resolving receptors, ALXR and ChemR23.

After establishing the time-course of the resolution response vs neuroinflammation during epileptogenesis, we designed interventional studies to boost and anticipate the endogenous pro-resolving response by focusing on ALXR agonists (Chapter 5). In fact, the activation of ALXR was previously shown to improve symptoms and disease outcomes in experimental models of neurological diseases such as neuropathic pain, neurotrauma, Alzheimer's disease and brain ischemia (Ayoub et al., 2008; Gavins et al., 2007; Hawkins et al., 2014; Luo et al., 2013; Medeiros et al., 2013).

4.2 SPECIFIC MATERIALS AND METHODS

Kainic acid model of SE

Mice were implanted with electrodes and guide cannulae as previously described in the General materials and methods, Section 3.3.1 and 3.4.1. SE was induced by intra-amygdala injection of kainic acid (0.3μg/0.2μl) (General materials and methods, Section 3.3.1 and 3.4.1). Our studies were performed at four time points post-SE: 2 h (acute phase of SE), 24 h, 72 h (epileptogenesis phase) and 7 days (spontaneous seizure onset). RT-qPCR was used to measure the hippocampal mRNA expression of IL-1β and TNF-α, pro-resolving receptors ChemR23 and ALXR, pro-resolving peptides IL-1Ra and Annexin A1, biosynthetic enzymes of lipid mediators LOX5 and LOX15 (n=7-10 mice) (Figure 4.1-4.4; General materials and methods, Section 3.7.1). Using different group of mice (n=8), the cellular expression of the same

molecules was studied by immunohistochemistry, 72 h after SE (Figure 4.1-4.4). In a separate group of mice (n=24), the levels of pro-resolving lipids LXA_4 and RvD1 were measured by ELISA (Figure 4.3; General materials and methods, Section 3.7.2). Control mice were implanted with electrodes and guide cannulae and injected with vehicle (n=23).

Immunohistochemistry

IL-1β, TNF-α, ChemR23, ALXR, Annexin A1 cellular expression in the hippocampus was assessed by immunohistochemistry 72 h post-SE (n=5) and compared to sham controls (n=5; General materials and methods, Section 3.6.1). In order to evaluate cell phenotype, additional experimental and sham mice (n=3 each group) were used for colocalization analysis of pro-inflammatory and pro-resolving molecules with NeuN, a neuronal marker, NPY, inhibitory interneurons marker, GFAP, astrocytic marker, and CD11b, microglial marker (Figure 4.1-4.4; General materials and methods, Section 3.6.1.3). ALXR signal was also co-localized with IL-1β, while the double-immunostaining of ChemR23 with IL-1β was not performed since the two primary antibodies were raised in the same host specie (goat). IL-1Ra immunohistochemistry was not performed due to the absence of a reliable and specific antibody for its detection in mice. Immunohistochemistry of ALXR and ChemR23 was performed in paraffin embedded brain specimens from patients who died within 5 days (n=4) or more than 7 days after SE (n=3) and in tissue surgically resected from patients affected by mesial temporal lobe epilepsy (MTLE, n=7) compared to autoptic control tissue from individuals who died with no neurological or infectious or autoimmune diseases (control, n=7) (Figure 4.5). For a detailed description of the immunohistochemical procedures in human tissue see General materials and methods, Section 3.6.3.

4.3 RESULTS

4.3.1 Neuroinflammation: induction of IL-1β and TNF-α

We measured IL-1β and TNF-α since they reflect neuroinflammation in the hippocampus during epileptogenesis induced by SE. Figure 4.1A,D shows a significant increase of IL-1β and TNF-α mRNA levels occurring between 2 h and 72 h after SE induction (n=7-10) compared to SHAM mice (n=7). At 7 days, IL-1β and TNF-α levels (n=7) persisted upregulated but were not statistically different from SHAM mice. RT-qPCR data were confirmed by immunohistochemistry (Figure 4.1B,E) performed 72 h post-SE. In control hippocampi (SHAM, n=8), IL-1β and TNF-α immunoreactivity was absent, while in mice exposed to SE (n=8) IL-1β and TNF-α staining was increased in activated GFAP-positive astrocytes as depicted by the colocalization analysis (Figure 4.1C,F). Cytokines were not detected in CD11b-positive microglia in both control and mice exposed to SE (Figure 4.1C,F).

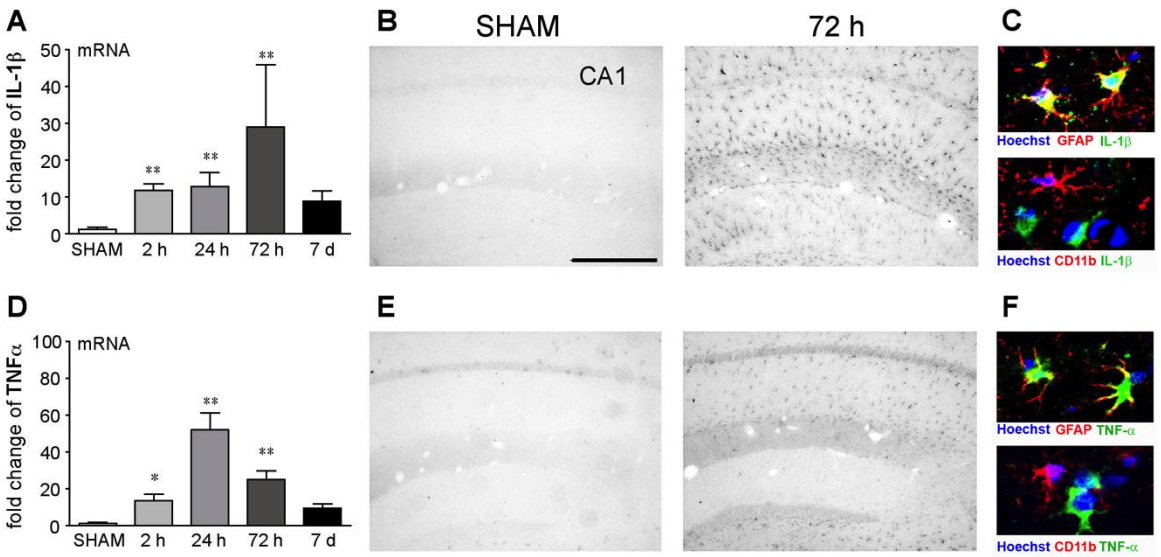


Figure 4.1. mRNA levels and immunohistochemical expression of IL-1β and TNF-α in the mouse hippocampus after SE. Bargrams show mRNA levels of IL-1β (A) and TNF-α (D) during epileptogenesis. RT-qPCR analysis was done from 2 h until 7 days post-SE (n=7-10). Reference genes are Mfsd5, Brap, Bcl2l13. Data are the mean ± s.e.m. *p<0.05, **p<0.01 vs SHAM by Kruskal Wallis test with Dunn's post-hoc correction. Panels B and E show representative photomicrographs of CA1 area in control mice (SHAM, n=8) and mice 72 h post-SE (n=8). We identified the cell types expressing IL-1β and TNF-α (in green) by double-immunostaining using specific astrocytic (GFAP, red) and microglial (CD11b, red) cell markers, as indicated in the high magnification panels (C and F). Co-localization signal is depicted in yellow. Hoecsht-positive nuclei are shown in blue. Scale bar 50 μm.

4.3.2 Pro-resolving peptides

We investigated the expression of IL-1Ra, a selective competitive antagonist of IL-1β receptor type 1. Figure 4.2A shows a significant up-regulation of mRNA levels between 24 h and 72 h post-SE (n=7-10) but not at 2 h (n=7) compared to control mice (SHAM, n=7). IL-1Ra levels declined by 7 days post-SE (n=7). Notably, IL-1Ra expression during epileptogenesis is significantly delayed as compared to IL-1β (Figure 4.1A).

Then, we assessed the expression of Annexin A1, an agonist of ALXR. Figure 4.2B,C shows the mRNA level of Annexin A1 (B) and the protein expression (C) evaluated by immunohistochemistry. mRNA levels significantly increased at 24 h and 72 h (n=7-10) but not at 2 h and 7 days (n=7) after SE compared to control mice (SHAM, n=7; Figure 4.2B). Annexin A1 was not detectable by immunohistochemistry in control sections (SHAM, n=8; Figure 4.2C)

while the specific staining was increased 72 h post-SE in GFAP-positive astrocytes (n=8; Figure 4.2C,D). Annexin A1 was not expressed by CD11b-positive microglia in control mice or after SE (Figure 4.2D). Thus, the cellular localization of Annexin A1 is similar to that of IL-1β and TNF-α. These data provide evidence that the up-regulation of pro-resolving peptides is delayed compared to onset of neuroinflammation and occurs in the same cell types.

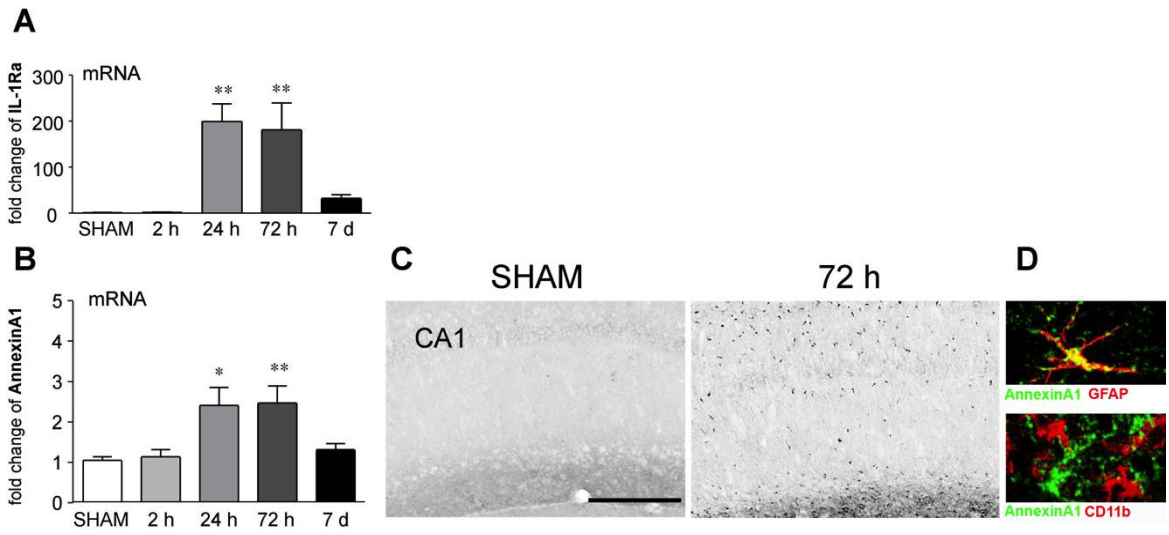


Figure 4.2. mRNA levels of IL-1Ra and Annexin A1 and immunohistochemical expression of Annexin A1 in the mouse hippocampus after SE. Bargrams show mRNA levels of IL-1Ra (A) and Annexin A1 (B) during epileptogenesis. RT-qPCR analysis was performed at various time-points after SE (n=7-10) and in SHAM controls (n=7). Reference genes are Mfsd5, Brap, Bcl2l13. Data are the mean ± s.e.m. *p<0.05, **p<0.01 vs SHAM by Kruskal Wallis test with Dunn's post-hoc correction. Panel (C) shows Annexin A1 expression at 72 h after SE (n=8) compared to SHAM (n=8). We identified the cell types expressing Annexin A1 (green) by double-immunostaining using specific astrocytic (GFAP, red) and microglial (CD11b, red) cell markers, as indicated in the high magnification panels (D). Co-localization signal is depicted in yellow. Scale bar 50 µm.

4.3.3 Biosynthetic enzymes and pro-resolving lipid mediators

Figure 4.3A,B shows mRNA levels of LOX5 and LOX15. Their expression increased significantly at 72 h after SE (n=7-10) compared to control mice (SHAM, n=7; Figure 4.3A,B). This is additional evidence of delayed activation of pro-resolving mechanisms during epileptogenesis. Figure 4.3C,D shows the levels of LXA_4 and RvD1, lipid ligands of the pro-resolving receptor ALXR, measured by ELISA. LXA_4 levels did not significantly differ from control mice (9.6±0.9

pg/mg hippocampus) at 72 h post-SE (9.0±0.4 pg/mg hippocampus; n=8 mice each group; Figure 4.3C). RvD1 levels were significantly increased by 1.6-fold 72 h after SE (12±0.6 pg/mg hippocampus, n=8) compared to control mice (7.5±1.3 pg/mg hippocampus, n=8; Figure 4.3D). A scheme of the biosynthetic pathway of the pro-resolving lipids is reported in Figure 1.5.3.

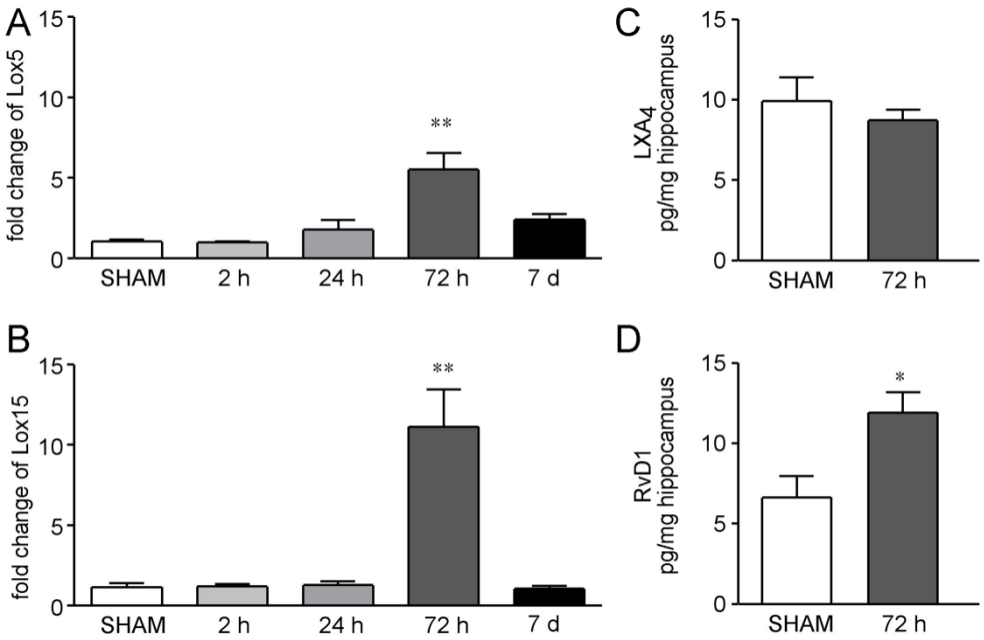


Figure 4.3. mRNA levels of LOX5 and LOX15 and lipid levels of LXA_4 and RvD1 in the mouse hippocampus after SE. Bargrams in panels (A) and (B) show mRNA levels of LOX5 (A) and LOX15 (B) at various time points after SE (n=7-10) and in SHAM controls (n=7). Reference genes are Mfsd5, Brap, Bcl2l13. Data are the mean ± s.e.m. **p<0.01 vs SHAM by Kruskal Wallis test with Dunn's post-hoc correction. Panels (C) and (D) show ELISA assay analysis of LXA_4 and RvD1 levels 72 h after SE (n=8) compared to SHAM (n=8). Data are mean ± s.e.m. *p<0.05 vs SHAM by Mann Whitney U test. A scheme of the biosynthetic pathway of the pro-resolving lipids is reported in Figure 1.5.3.

4.3.4 Pro-resolving receptors

After the characterization of lipid and peptide mediators of resolution, we investigated the expression of pro-resolving receptors ALXR and ChemR23 during epileptogenesis in the hippocampus of mice exposed to SE vs their respective controls (SHAM). Figure 4.4A shows a significant induction of ALXR mRNA at 24 h and 72 h (n=7), but not 2 h and 7 days, after SE induction compared to SHAM (n=7-10 each group). Accordingly, immunohistochemical analysis

shows that ALXR was increased 72 h post-SE (Figure 4.4B,C,D). In SHAM, ALXR immunoreactivity was confined to CA1 and CA3 pyramidal neurons and granule cells of the dentate gyrus (DG) (thick green lines in panel C). Receptor expression decreased in pyramidal and DG neurons but increased in glial cells 72 h after SE (thin green lines and brown spots in panel C). We detected a significant increase in the number of ALXR positive neurons and astrocytes 72 h post-SE by quantitative analysis of the staining performed in DAB coloured sections (General materials and methods, Section 3.6.1.2): number of cells in CA1 area, SHAM (n=5), 32.1 ± 6.7; 72 h post-SE (n=5), 160.2 ± 8.6 ($p<0.05$).

Co-localization analysis showed that ALXR was expressed in NeuN positive neurons in sham mice (Figure 4.4B,D). After SE, the receptor was induced in GFAP-positive astrocytes but not in CD11b-positive microglia (Figure 4.4B,D). Moreover, ALXR co-localized with IL-1β in astrocytes (Figure 4.4D), indicating that the resolution process was activated in the cells generating also the neuroinfammatory molecules.

Figure 4.4E shows a significant induction of ChemR23 mRNA 72 h post-SE (n=10) but not at the other investigated time points compared to SHAM (n=7). In sham mice, ChemR23 was expressed predominantly by interneurons localized in the CA1-3 pyramidal layers and in the hilus (green spots in panel G) as well as by neurons of the DG (green line in panel G). Receptor expression increased in astrocytes (Figure 4.4F,H) in the strata radiatum and moleculare (brown spots in panel G) 72 h after SE while its neuronal expression did not change.

The number of ChemR23 positive neurons and astrocytes was significantly up-regulated 72 h post-SE as depicted by a quantitative analysis of the staining performed in DAB coloured sections: number of cells in CA1 area, SHAM (n=5), 19.4 ± 3.6; 72 h post-SE (n=5), 59.0 ± 12.6 ($p<0.05$).

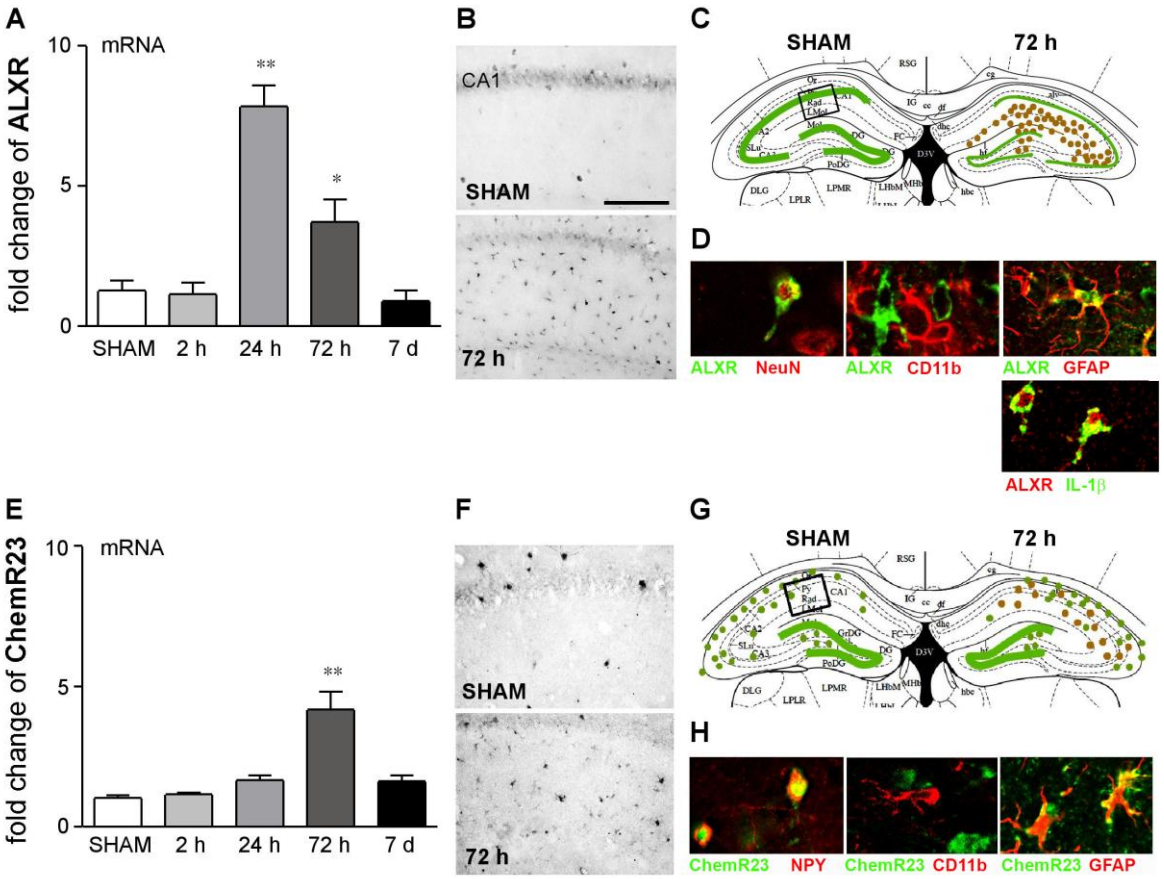
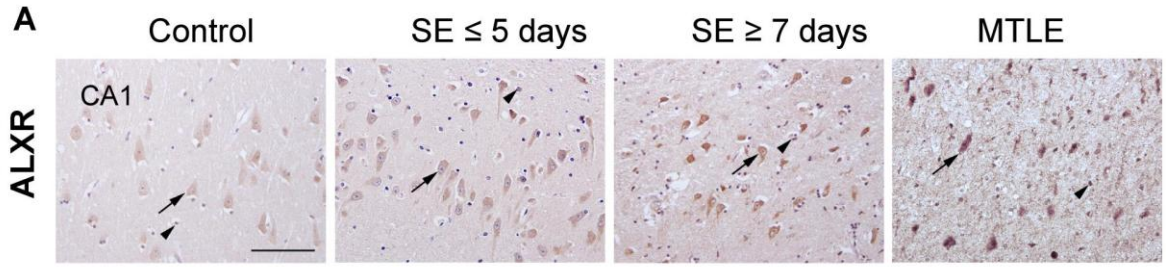


Figure 4.4. mRNA levels and cell type-specific expression of ALXR and ChemR23 in the mouse hippocampus after SE. Bargrams show mRNA levels of ALXR (A) and ChemR23 (E) at various time points after SE (n=7 each group) and in SHAM control (n=7). Reference genes are Mfsd5, Brap, Bcl2l13. Data are the mean ± s.e.m. *p<0.05, **p<0.01 vs SHAM by Kruskal Wallis test with Dunn's post-hoc correction. Panels (C) and (G) show a representative scheme of the ALXR and ChemR23 immunoreactivity in hippocampus of mice 72 h post-SE and in SHAM controls (n=8 mice each group). Green lines represent CA1-3 pyramidal neurons and DG immunopositive neurons, green dots represent interneurons in hilus and in the molecular layer. For both receptors, brown dots are representative of cells with glial morphology. The boxes in both schemes show the CA1 area chosen for representative images reported in panels (B) and (F) where we quantified ALXR and ChemR23 positive cells. Panels (B) and (F) represent the immunoreactivity of ALXR or ChemR23 in CA1 area of SHAM (n=8) and mice 72 h post-SE (n=8). Cell types expressing ALXR and ChemR23 (green) were identified by double-immunostaining using specific astrocytic (GFAP, red), microglial (CD11b, red), neuronal (NeuN, red) and interneuronal (NPY, red) markers, as indicated in the high magnification panels. ALXR (red) co-localized also with IL-1β (green). The double-immunostaining of ChemR23 with IL-1β was not performed since the two primary antibodies were raised in the same host specie (goat). Co-localization signal is depicted in yellow. Scale bar 50 µm.

4.3.5 Pro-resolving receptors in the hippocampus of patients after SE and with mesial temporal lobe epilepsy (MTLE)

To validate our findings in the mouse model in human brain tissue, we evaluated ALXR and ChemR23 expression in hippocampal specimens from patients who died after SE as well as in hippocampi surgically resected in patients affected by MTLE (Figure 4.5). In autoptic control tissue (n=6), ALXR (Figure 4.5A) and ChemR23 (Figure 4.5B) immunoreactivity was observed in scattered CA1 and CA3 pyramidal cells, granule cells of the DG and hilar interneurons (arrows). Pro-resolving receptors were induced after SE (SE ≥5 days, n=4; SE ≥7 days, n=3; Figure 4.5A,B) as well as in MTLE tissues (n=7, Figure 4.5A,B) in glial cells (arrowheads). Semi-quantitative evaluation of the specific staining is reported in the annexed table (C). Human tissue evidence confirmed the increased expression of these receptors in glial cells as we reported in mice during epileptogenesis.



C

ALXR	CA1 neuron	CA1 glia	ChemR23	CA1 neuron	CA1 glia
Control (n=6)	6	1	Control (n=6)	6	1
SE ≤ 5 d (n=4)	7.5 (6-9)	6 (2-9)*	SE ≤ 5 d (n=4)	7.5 (6-9)	6 (1-9)
SE ≥ 7 d (n=3)	9 (6-9)	9 (4-9)*	SE ≥ 7 d (n=3)	9 (6-9)	9 (4-9)*
MTLE (n=7)	9 (6-9)	9 (2-9)*	MTLE (n=7)	9 (2-6)*	9 (2-6)*

Figure 4.5. Cell type-specific expression of ALXR and ChemR23 in the hippocampus of patients died after SE and of patients with mesial temporal lobe epilepsy (MTLE). Panels (A) and (B) show ALXR and ChemR23 immunostaining in CA1 area from autoptic control tissue (n=6) and patients who died within 5 days after SE (SE ≤5 days, n=4) or more than 7 days after SE (SE ≥7 days, n=3) or MTLE patients (MTLE, n=7). We identified a prevalent neuronal expression of the receptors (arrows) in controls while we detected an increased glial expression (arrowheads) after SE and MTLE patients. Table C reports semi-quantitative analysis of staining which was done by calculating the immunoreactivity score: intensity of staining x frequency of stained cells. Intensity: 0=negative, 1=weak, 2=moderate, 3=strong. Frequency: 1≤10%, 2=11-50%, 3≥50%. ***** $p<0.05$ vs control by Mann Whitney test. Scale bar 100 µm.

4.4 DISCUSSION

Neuroinflammation is activated following SE induced by intra-amygdala kainate as previously shown in other models of epileptogenesis induced by electrical stimulation or by chemoconvulsants (Maroso et al., 2010; Pauletti et al., 2017; Walker et al., 2017). As demonstrated by temporal profile of the induction of pro-inflammatory cytokines IL-1β and TNF-α, neuroinflammation was rapidly increased after SE onset (i.e. 2 h) and persisted elevated during the epileptogenesis phase until the time of spontaneous seizure onset. Notably, IL-1β and TNF-α are endowed of ictogenic properties and promote seizure recurrence in experimental models (De Simoni et al., 2000; Eriksson et al., 1998; Maroso et al., 2011b; Shandra et al., 2002; Vezzani et al., 2000).

Acute inflammation normally resolves by active resolution mechanisms which initiate in the first few hours after an inflammatory response begins (Serhan et al., 2005). Our data clearly showed that SE evokes a neuroinflammatory response already evident by 2 h from the insut in the absence of active resolution mechanisms such as the pro-resolving peptide mediators, biosynthetic enzymes of lipid mediators and their cognate receptors.

In particular, lipoxygenase enzymes, responsible for the conversion of AA or n-3 polyunsaturated fatty acids, such as EPA and DHA in pro-resolving lipid mediators, were upregulated only at 72 h after SE indicating a delayed and transient activation of the pro-resolving response. Moreover, an increase in biosynthetic enzymes should be accompanied by a concomitant elevation in pro-resolving lipids. This is true for RvD1, lipid ligand of ALXR, which is increased by 2-fold on average during epileptogenesis. However, levels of LXA_4, another lipid ligand of ALXR, were similar to control as reported also in tissue from AD affected patients (Wang et al., 2015b). We cannot exclude that LXA_4 quantification at later time points would have shown changes in its level.

The last step in the characterization of the pro-resolving response, is represented by the pro-resolving receptors, ALXR and ChemR23. Their neuronal expression in control tissue may have

a neuromodulatory function, as reported for IL-1R1 (Friedman, 2001; Viviani et al., 2003). Indeed, cytokine receptors have physiological functions that include neurite outgrowth, neurogenesis, neuronal survival, synaptic pruning during brain development and they regulate the strength of synaptic transmission and synaptic plasticity (Marin and Kipnis, 2013).

After SE, ALXR and ChemR23 expression was increased in astrocytes but with a delay as compared to the onset of neuroinflammation. Induction of these receptors during epileptogenesis may indicate a compensatory response evoked by the reduced availability of specialized pro-resolving mediators. Moreover, ALXR can be activated also by endogenous molecules other than pro-resolving lipids (see Section 5.4).

Regarding ChemR23, the function of its putative agonist chemerin is unclear, since it can exert both pro-resolving and pro-inflammatory roles (Mariani and Roncucci, 2015). Prochemerin can be converted into the full active form by proteolytic cleavage and removal of the last inhibitory amino acids in the C-terminal region. The multiple cleavage sites in the C-terminal domain generate chemerin peptide isoforms with different bioactivity profiles. The various chemerin isoforms by acting on ChemR23 induce chemotaxis of ChemR23-expressing immune cells at the beginning of an inflammatory response, while during resolution of inflammation the chemerin/ChemR23 axis inhibits the release of pro-inflammatory mediators (Mariani and Roncucci, 2015).

We found a specific staining of ALXR, ChemR23 and Annexin A1 in astrocytes as reported also for the pro-inflammatory cytokines. Moreover, we demonstrated by co-localization analysis that ALXR and IL-1β were expressed by the same astrocytic cell population. This data suggests that neuroinflammation and resolution are two processes induced in the same cell population. In accordance with our results, the expression of lipoxygenase LOX15 was increased in AD hippocampus in both astrocytes and microglia (Wang et al., 2015b).

Up-regulation of the pro-resolving response likely represents a homeostatic attempt to compensate for the increased neuroinflammatory response induced by SE. Since we did not measure the pro-resolving mediators between 2 and 24 h post-SE, we cannot exclude that

resolution activates during the first 24 h after injury. The available data indicates that delayed and transient activation of resolution mechanisms may therefore play a role in determining a persistent neuroinflammation which may become detrimental for the tissue. Indeed, increasing IL-1Ra levels by pharmacological interventions with anakinra (the human recombinant protein) delayed seizure onset and reduced their frequency and generalization in experimental models (Maroso et al., 2011b; Ravizza and Vezzani, 2006; Vezzani et al., 2000, 2011c) and determined therapeutic effects in pediatric forms of phamacoresistant epilepsy (Jyonouchi, 2016; Kenney-Jung et al., 2016).

Our data report novel evidence of activation of resolution mechanisms in patients who died after SE and in people with chronic epilepsy thus indicating that these mechanisms do occur also in humans. These findings therefore raise the possibility that boosting the resolution of neuroinflammation may have a therapeutic potential for epilepsy.

CHAPTER 5 - PRO-RESOLVING TREATMENTS

DURING EPILEPTOGENESIS

5.1 INTRODUCTION

Neuroinflammation during epileptogenesis occurs rapidly and persists for days after the epileptogenc insult which is likely mediating its detrimental effects on neuronal excitability and survival (Vezzani et al., 2013). In chapter 4, we investigated the resolution mechanisms during epileptogenesis and demonstrated they are delayed as compared to the onset of neuroinflammation. Therefore, we hypothesized that to anticipate and boost the resolution response after the inciting event may represent a therapeutic option to counteract the pathogenic effects of neuroinflammation. We chose therefore a treatment protocol for anticipating and incrementing the activation of the pro-resolving system in order to counteract neuroinflammation during early epileptogenesis. We selected analogues of pro-resolving peptides and lipids which showed positive effects on neuroinflammation and pathology in experimental models of neuropathic pain, neurotrauma, ischemia and AD (Gavins et al., 2007; Hawkins et al., 2014; Luo et al., 2013; Martini et al., 2016; Medeiros et al., 2013; Svensson et al., 2007).

In order to design the long-term pharmacological study on epileptogenesis, we first tested and compared the effects of three different molecules administered post-SE on animal's body weight- which is decreased following SE- and on neuroinflammation by measuring IL-1β mRNA hippocampal level. I tested two molecules that activate ALXR: (1) a stable synthetic analogue of the lipid mediator LXA_4 (BML111) and (2) the N-terminal derived peptide of Annexin A1 (Ac2-50). The third molecule was a methylated form of $PD1_{n\text{-}3DPA}$ ($PD1_{n\text{-}3DPA}ME$) whose specific receptor and mechanisms of action have not been elucidated yet (Hansen et al., 2017), but its levels were significantly increased during epileptogenesis as assessed by lipidomic analysis. Moreover, PD1 showed antiepileptogenic effects after kindling and pilocarpine-induced SE in rats and mice (Musto et al., 2011, 2015).

5.2 SPECIFIC MATERIALS AND METHODS

Pharmacological treatments

Mice were implanted with electrodes and guide cannulae as previously described in the General materials and methods, Section 3.3.1 and 3.4.1. Mice were exposed to SE as previously described then were assigned randomly to treatment or vehicle groups (n=6-9 each group). Drugs or their vehicles were injected for three consecutive days starting 1 hour after SE onset to encompass the epileptogenesis phase preceding disease onset, then the treatment was stopped (Figure 5.1). The treatment protocol was chosen based on our previous data on the time of induction and the persistence of pro-resolving mechanisms (Chapter 4). The following drugs were used:

- **BML111**: is a derivative of LXA_4, a methyl ester of 5(S),6(R),7-Trihydroxyheptanoic acid (Sigma-Aldrich, #SML0215), which is more resistant to enzymatic inactivation than the endogenous isoform. BML111 (0.1 µg in 1 µl of NaCl 0.9% + EtOH 0.1%) was injected icv twice/day in 1.75 µl/site bilaterally (n=7 mice). A similar treatment schedule using LXA_4ME (a synthetic stable analog of LXA_4) was reported to be effective in reducing inflammatory cytokines triggered by icv injection of Aβ1-42 (Wu et al., 2011).

- **Ac2-50**: is constituted by 50 amino acids derived from the N-terminal portion of Annexin A1; residues from 2 to 26 are essential for the biological activity of the protein. Ac2-50 (0.1 µg in 1 µl 50 mM PBS, pH 7.4) was injected icv in 1 µl/site twice on the first day post-SE and once daily thereafter (n=7 mice). Since previous data on icv injection were lacking we empirically used the highest soluble concentration that we could inject.

- **$PD1_{n\text{-}3DPA}ME$**: stable analogue of $PD1_{n\text{-}3DPA}$ with an additional methyl group. $PD1_{n\text{-}3DPA}ME$ (0.2 µg/µl in 50 mM PBS, pH 7.4) was injected icv in 1 µl/site and twice/daily (n=6 mice). This dose was based on previous evidence in the literature (Musto et al., 2011) and on our evidence that dose 10 times lower was not effective on neuroinflammation. $PD1_{n\text{-}3DPA}ME$ was aliquoted in ethanol and stored at -80°C in the dark until use. Immediately prior to administration in mice,

ethanol was evaporated using a gentle stream of nitrogen. Then the lipid mediator was suspended in 50 mM PBS (pH 7.4), placed in a water bath sonicator for 10 seconds and vortexed for 30 seconds.

Control mice were similarly exposed to SE but injected with the corresponding vehicles (Saline, n=7-9). SHAM mice were implanted with electrodes and guide cannulae and received saline instead of kainic acid and the drug's vehicle (n=8-9). EEG recordings were done continuously for 24 h before (baseline) and for 24 h after SE induction in order to evaluate SE severity by EEG analysis (Figure 5.1). We evaluated SE duration and the number of spikes during the first 12 h using Clampfit 9.0 program (Axon Instruments, Union City, CA, U.S.A.). SE ended when spike frequency was lower than 1 Hz (1 spike/sec, General materials and methods, Section 3.4.4).

All mice were weighted between 8:00 and 9:00 a.m. at 24 h (baseline value) before SE induction, then 24 h, 48 h and 72 h post-SE (Figure 5.1, 5.5 and 5.8). Mice were treated with BML111, Ac2-50 or $PD1_{n\text{-}3DPA}ME$ for 3 days post-SE and 2 hours after the last injection of the pro-resolving mediator (i.e. 72 h post-SE) mice were sacrified and their hippocampi ipsilateral to the injected amygdala were harvested for RT-qPCR analysis (Figure 5.1, 5.4 and 5.7; General materials and methods, Section 3.7.1).

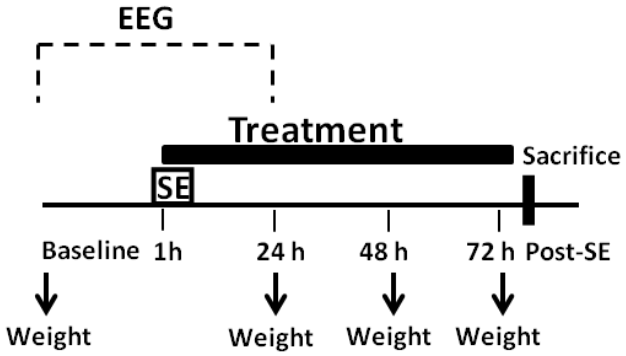


Figure 5.1. Experimental design in SE-exposed mice treated with BML111 or Ac2-50 or PD1$_{n\text{-}3DPA}$ME during epileptogenesis. The scheme reports the treatment schedule post-SE, EEG recording time and the time points at which mice were weighted during the experiment. EEG recordings were done continuously 24 h before (Baseline) and for 24 h after SE induction. One hour after SE onset, mice were randomized into treatment or vehicle groups (n=7 BML111, n=7 Ac2-50, n=6 PD1$_{n\text{-}3DPA}$ME mice and n=7-9 saline). Treatment protocol is described for each molecules in the text. Control mice (n=7-9) were exposed to SE and injected with respective vehicle. Mice were sacrified at the end of the experiment for RT-qPCR analysis (Sacrifice). Data related to this protocol are reported in Figure 5.3, 5.4, 5.6 and 5.7.

Two additional groups of mice (n=9-11) were injected with PD1$_{n\text{-}3DPA}$ME for 4 days post-SE induction in order to cover the average latency phase before disease onset that in this model is about 5 days.

A first group of mice was used for the evaluation of non spatial recognition memory by Novel Object Recognition Test (NORT; General materials and methods, Section 3.5 and Figure 3.4). This test was performed for 3 consecutive days starting at 48 h after SE induction.

At the end of the last session mice were sacrified (i.e. 96 h after SE; Figure 5.2 and 5.8). They were perfused and their brains were removed from the skull, frozen and stored at -80°C for neurodegeneration analysis by Fluoro-Jade staining (in progress).

EEG recordings were done continuously for 24 h before (baseline) and for 24 h after SE induction (Figure 5.2) in order to evaluate SE duration and the number of spikes during the first 12 h (General materials and methods, Section 3.4.4).

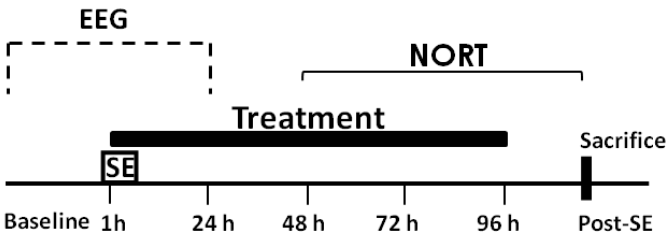


Figure 5.2. Experimental design in SE-exposed mice treated with $PD1_{n\text{-}3DPA}$ME and assessed for recognition memory. The scheme reports the treatment schedule post-SE and the EEG recording time. EEG recordings were done continuously 24 h before (Baseline) and for 24 h after SE induction to assess SE duration. One hour after SE onset, mice were randomized into treatment (n=11) or vehicle groups (n=12). Mice were tested for recognition memory by NORT. Mice were sacrified at the end of the experiment (i.e. 96 h after SE) for analysis of neurodegeneration (Sacrifice) by FluroJade staining. Data related to this protocol are reported in Figure 5.8.

A second group of mice was used for the evaluation of the effect of $PD1_{n\text{-}3DPA}$ME on epileptogenesis (Figure 5.9). Mice were EEG recorded until the onset of two spontaneous seizures and for 2 weeks thereafter in order to assess the effect of the treatment on seizure frequency and duration during the early stages of the disease. In order to test long-term effect on spontaneous seizures, mice will be EEG recorded at 2 months post-epilepsy onset (in progress). Also in this experiment SE duration and the number of spikes during the first 12 h were evaluated (General materials and methods, Section 3.4.4) .

In each of the two set of experiments, one group of mice was exposed to SE but injected with drug's vehicle (n=12 mice each experiment); SHAM mice (n=9-18) were implanted with electrodes and guide cannulae and injected with vehicles.

Lipidomic analysis

One group of mice (n=8) was exposed to SE and sacrified at 72 h after SE onset to measure lipid mediators by LC/MS-MS (Figure 5.5, Table 5.1; General materials and methods, Section 3.7.3). SHAM mice (n=8) implanted with electrodes and guide cannulae and vehicle injected served as controls.

5.3 RESULTS

5.3.1 Effects of a synthetic stable analogue of LXA_4 or Annexin A1 N-terminal derived peptide on animal weight and IL-1β level

BML111 and Ac2-50 or their vehicles were injected in mice for 3 consecutive days starting 1 h after SE onset. In order to check whether the drug was modifying the initial insult, SE duration and spike number were reckoned. SE duration was not significantly modified by the treatments (Saline, 4.8±1.0 h; BML111, 5.8±1.5 h; Ac2-50, 9.0±3.0 h) although there was a trend towards an increased SE duration induced by Ac2-50. The number of spikes during 12 h of SE was not modified by the treatment (Saline, 47500 ± 8412; BML111, 50910 ± 9696; Ac2-50, 55609 ± 18996). A decrease in weight is observed in SE-exposed mice, therefore a gain in weight is a measure of faster post-injury recovery. Figure 5.3 shows that SE-exposed mice injected with vehicles (n=9) had a significant 14% reduction in weight as compared to their baseline value and to sham mice (n=8). Mice treated with each of the two ALXR agonists (n=7 mice each group) lost weight during epileptogenesis similarly to mice exposed to SE and injected with vehicle.

Figure 5.4 shows a significant induction of IL-1β mRNA 72 h after SE in mice treated with vehicle (Saline, n=9) as compared to control mice (SHAM, n=8). The up-regulation of IL-1β was not affected by administration of either BML111 (n=7) or Ac2-50 (n=7).

Overall, these two drugs activating ALXR failed to reduce neuroinflammation and weight loss induced by SE. The data indicate that either the ALXR activated pathways are not sufficient to resolve neuroinflammation during epileptogenesis or that the treatment protocol did not attain therapeutic drug concentrations in the hippocampus, probably due to limited solubility of the two compounds in the small volume of icv injections.

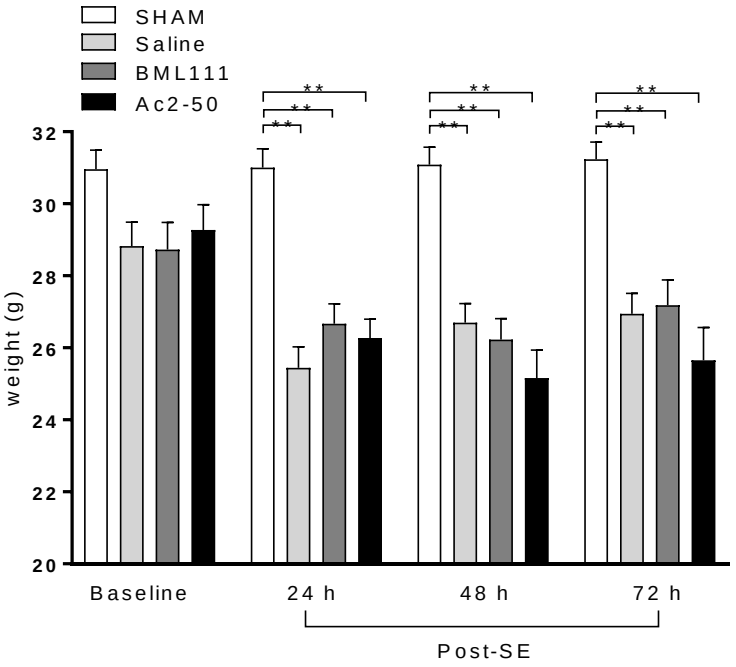


Figure 5.3. BML111 or Ac2-50 administered during epileptogenesis did not rescue weight loss in SE-exposed mice. Bargrams report mice weight at baseline and at three consecutive time points during epileptogenesis (n=7-9) in the vehicle (saline) and treatment groups (BML111 and Ac2-50) compared to SHAM mice (n=8). Data are the mean ± s.e.m. **p<0.01 by Kruskal Wallis test with Dunn's post-hoc correction.

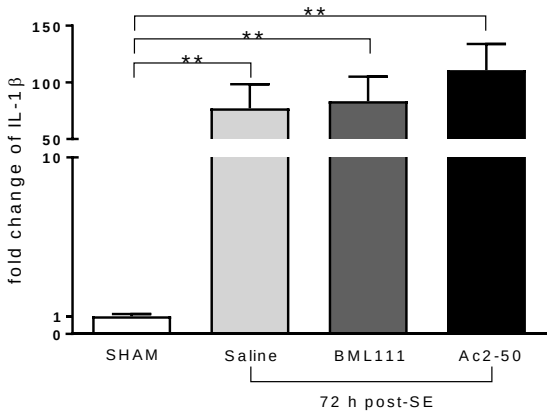


Figure 5.4. Lack of effect of BML111 or Ac2-50 treatment on IL-1β level in SE-exposed mice. Bargrams show fold-change of IL-1β mRNA levels in the hippocampus 72 h after SE in mice treated with vehicle (Saline, n=9), BML111 (n=7) or Ac2-50 (n=7) compared to control mice (SHAM, n=8). Reference genes are hprt1, sv2b, pgk1. Data are the mean ± s.e.m. **p<0.01 by Kruskal-Wallis test with Dunn's post-hoc correction.

5.3.2 Lipidomic analysis

We investigated the profile of pro-resolving lipid mediators during epileptogenesis (i.e., 72 h after SE induction) in order to find a new potential target for pharmacological intervention.

Lipidomic analysis was done in the frame of collaboration with Dr. Jesmond Dalli (Lipid Mediator Unit, William Harvey Research Institute, London, UK). The analysis was performed by liquid chromatography followed by mass spectrometry in experimental mice (n=8) and in their controls (SHAM, n=8). Figure 5.5 and Table 5.1 show (1) an up-regulation of DHA- and n-3DPA-derived neuroprotectins (PD1 and $PD1_{n\text{-}3DPA}$) and of E-series resolvins; (2) a down-regulation of AA-derived Lipoxins (5S, 15S-diHETE, 15R-LXA_4) and of DHA- and n-3DPA-derived D-series resolvins (RvD4, $RvD2_{n\text{-}3DPA}$, $RvD5_{n\text{-}3DPA}$). Notably, the lipid mediator $PD1_{n\text{-}3DPA}$ was upregulated by 20-fold in the hippocampus of SE-exposed mice (Table 5.1). This protectin does not derive from DHA but from n-3 docosapentaenoic acid (n-3DPA), an intermediate in the metabolic pathway of DHA (Ferdinandusse et al., 2001; Hansen et al., 2017). DPA is detected in plasma, retina and heart at levels comparable to those of EPA and DHA, and it is the precursor of the synthesis of some pro-resolving lipid mediators.

Lipidomic analysis (Table 5.1) confirmed that LXA_4 levels were not modified following SE as measured by ELISA (Section 4.3.3), whereas RvD1 levels raised by 50% although not significantly (Table 5.1), at variance with ELISA data (Figure 4.3).

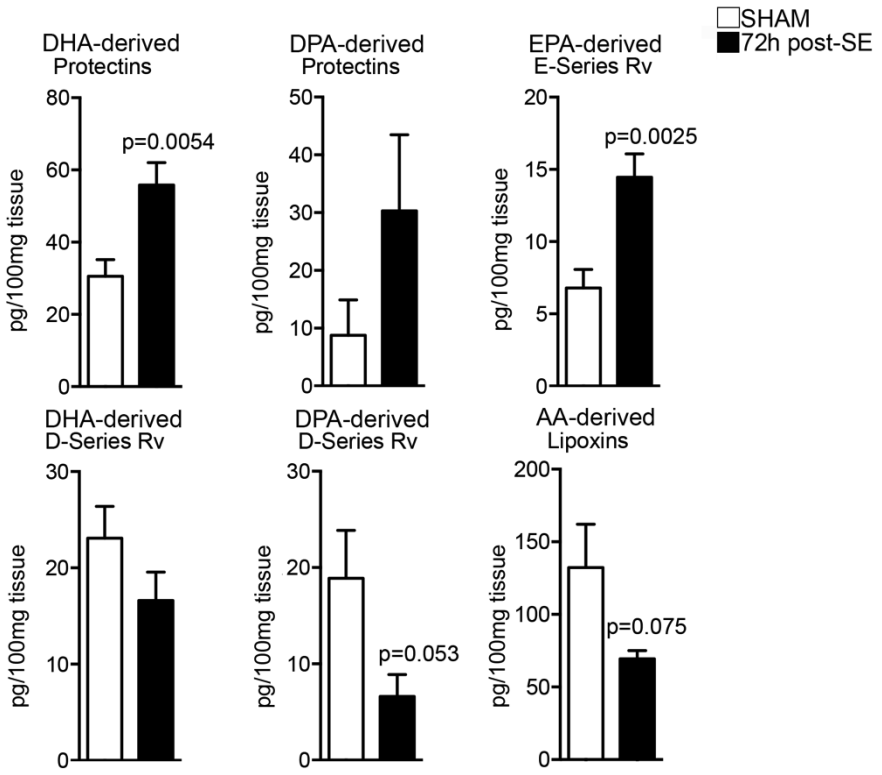


Figure 5.5. Lipidomic analysis of the hippocampus during epileptogenesis. Bargrams report levels of various lipid mediators 72 h after SE (n=8) compared to SHAM mice (n=8). Data are the mean ± s.e.m. p values are reported in the figure by t test.

Table 5.1. *Levels of lipid mediators in the hippocampus of mice during epileptogenesis (i.e. 72 h post-SE) vs control mice (SHAM).*

Lipid mediator	SHAM	72 h post-SE
PD1	17.9 ± 2.3	31.2 ± 7.9
$PD1_{n\text{-}3DPA}$	1.2 ± 0.9	24.2 ± 8.2*
RvD1	0.4 ± 0.1	0.6 ± 0.1
RvD4	8.5 ± 1.9	1.4 ± 0.3*
$RvD2_{n\text{-}3DPA}$	17.4 ± 5.5	4.3 ± 1.7*
$RvD5_{n\text{-}3DPA}$	1.1 ± 0.7	1.8 ± 0.9*
5S, 15S-diHETE	74.2 ± 15.6	42.2 ± 5.4*
15R-LXA_4	44.0 ± 16.9	26.3 ± 14.4
LXA_4	1.5 ± 0.9	2.6 ± 2.0

*Changes in lipid mediator levels (pg/100 mg tissue) were assessed by LC-MS/MS. The table reports significant up-regulation (PD1 and $PD1_{n\text{-}3DPA}$) or down-regulation (RvD4, $RvD2_{n\text{-}3DPA}$, $RvD5_{n\text{-}3DPA}$) of specific lipid mediators. No changes were observed in LXA_4 confirming the results obtained by ELISA (Section 4.3.3) and in RvD1 which was found increased by ELISA. Data are the mean ± s.e.m. *p<0.05 by t test.*

5.3.3 Effects of a synthetic stable analogue of $PD1_{n\text{-}3DPA}$ on animal weight and IL-1β level

Since $PD1_{n\text{-}3DPA}$ was prominently upregulated during epileptogenesis, we explored whether further incrementing $PD1_{n\text{-}3DPA}$ levels would reduce neuroinflammation. SE duration was not modified by the pro-resolving mediator analogue (Saline, 7.7 ± 1.2 h; $PD1_{n\text{-}3DPA}$ME, 6.6 ± 1.5 h). The number of spikes during 12 h of SE was not modified by the treatment (Saline, 52075 ± 7581; $PD1_{n\text{-}3DPA}$ME, 42043 ± 11428). Representative EEG tracings of spiking activity during SE in the two experimental groups are depicted in Figure 5.6A. $PD1_{n\text{-}3DPA}$ME or its vehicle were injected in mice for 3 days starting 1 h after SE induction. As previously shown (Figure 5.3), mice exposed to SE and injected with vehicle (n=9) significantly lost weight after SE compared to their baseline value and to sham mice (n=8; Figure 5.6B). Mice treated with the methylated form of $PD1_{n\text{-}3DPA}$ (n=7) recovered from their weight loss more rapidly than vehicle injected SE mice (Figure 5.6B).

Figure 5.7 shows a significant induction of IL-1β mRNA levels 72 h after SE in mice treated with saline (SE+Saline, n=7) compared to SHAM mice (n=8). The increase in IL-1β was drastically reduced, although not fully prevented, by $PD1_{n\text{-}3DPA}$ME (n=6), indicating that the treatment protocol induced a pro-resolving anti-inflammatory response.

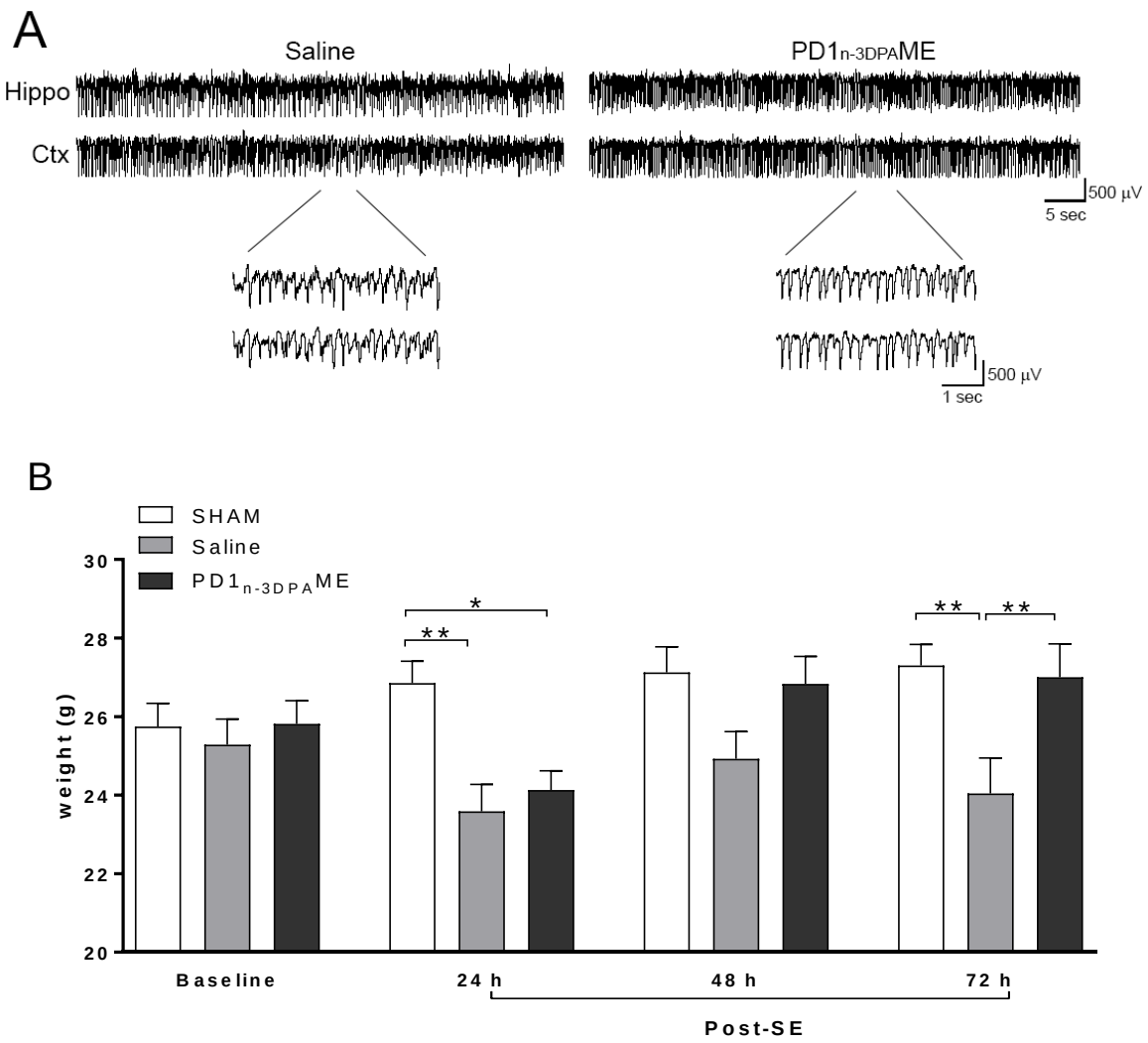


Figure 5.6.* $PD1_{n\text{-}3DPA}ME$ *administered during epileptogenesis rescued weight loss in SE-exposed mice without modifying SE. Panel A: representative EEG tracings depicting spike activity occurring during SE as recorded in the right hippocampus (Hippo) and in the left cortex (Ctx) of mice treated with saline (left) or $PD1_{n\text{-}3DPA}ME$ (right). Bargrams in panel B report the weight of mice at baseline and at three consecutive time points during epileptogenesis in the saline (n=7) and treatment (n=6) groups as compared to SHAM mice (n=9). Data are the mean ± s.e.m. *p<0.05; **p<0.01 by Kruskal Wallis test with Dunn's post-hoc correction.

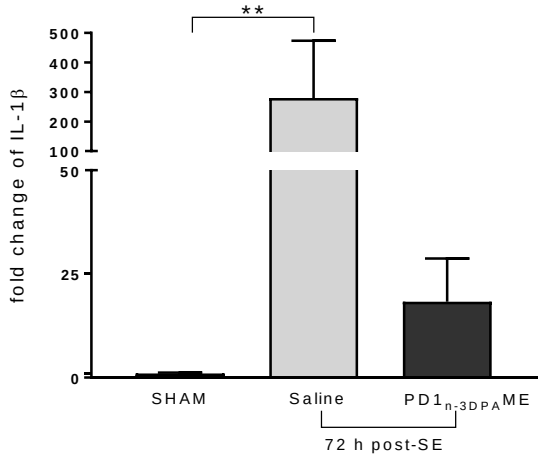


Figure 5.7. PD1$_{n-3DPA}$ME treatment reduced IL-1β level in SE-exposed mice. Bargrams show mRNA levels of IL-1β in hippocampus 72 h after SE in mice treated with vehicle (Saline, n=7) or PD1$_{n-3DPA}$ME (n=6) compared to control mice (SHAM, n=9). Reference genes are hprt1, sv2b, pgk1. Data are the mean ± s.e.m. ** p<0.01 by Kruskal Wallis test with Dunn's post-hoc correction.

5.3.4 Effect of the treatment with PD1$_{n-3DPA}$ME on recognition memory

Because of these initial positive results, the effect of PD1$_{n-3DPA}$ME was tested on cognitive deficits developing during epileptogenesis. PD1$_{n-3DPA}$ME (n=11) or vehicle (n=12) were injected in mice for 4 days starting 1 h after SE induction. SE was not modified by the treatment as previously reported (Section 5.3.3). Impairment in cognitive functions after seizures and during epileptogenesis has been reported in different experimental models (Iori et al., 2013; Pascente et al., 2016; Pearson et al., 2014; Rojas et al., 2015). We performed NORT test as a measure of non spatial recognition memory. During the recognition phase of NORT (Figure 3.4 and 5.8A), control mice (n=18) spent significantly more time exploring the novel object, as compared to the familiar one (approximately 70% and 30% of total exploration time respectively), thus yielding a discrimination index of 0.37±0.03. By contrast, mice injected with saline and exposed to SE, spent equal time exploring previously presented and novel objects; hence the discrimination index was 0.13±0.05. Mice exposed to SE and injected with PD1$_{n-3DPA}$ME spent more time exploring the novel object thus yielding a discrimination index of 0.34±0.05 (Figure 5.8A) similar to control mice.

The reduced object exploratory behavior induced by SE was not a result of changes in spontaneous motor activity, since the distance covered and the velocity of mice in the open field were not affected by SE or by the treatment (SHAM, 3061±192 cm and 5.1±0.3 cm/s; Saline, 3364±574 cm and 5.3±0.6 cm/s; $PD1_{n\text{-}3DPA}ME$, 3890±613 cm and 6.9±1.2 cm/s). As depicted in Figure 5.8B, the three groups of mice spent equal time (approximately 50%) exploring the two identical objects, therefore excluding that the reduced discrimination index in SE-exposed mice was a consequence of reduced objects' exploration time during the familiarization phase. Thus, the data indicate that there is an impairment in the long-term memory encoding in SE mice which was rescued by $PD1_{n\text{-}3DPA}ME$.

In conclusion, the pro-resolving treatment with $PD1_{n\text{-}3DPA}ME$ rescued cognitive deficits in a non spatial recognition memory test probably by reducing neuroinflammation during epileptogenesis. In accordance, there is supporting evidence that neuroinflammatory mediators, and in particular IL-1β, mediate cognitive deficits in rodents (Goshen and Yirmiya, 2009; Goshen et al., 2007; Mazarati et al., 2011).

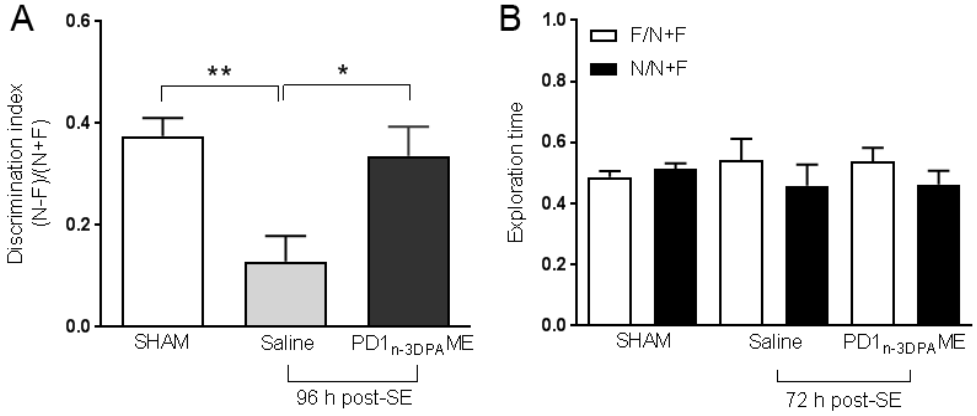


Figure 5.8. $PD1_{n\text{-}3DPA}ME$ treatment rescued cognitive deficit in SE-exposed mice. Bargrams in panel A show the discrimination index (the ratio between the difference in time spent with the novel and the familiar object (N-F) and the sum of total exploration time (N+F)**.** The test was performed 96 h after SE in mice treated with saline (n=12, Saline) or $PD1_{n\text{-}3DPA}ME$ (n=11, $PD1_{n\text{-}3DPA}ME$) as compared to SHAM mice (n=18). Data are the mean ± s.e.m. * $p<0.05$; ** $p<0.01$ vs Sham by one-way ANOVA with Tukey's post-hoc test. Bargrams in panel B show exploration time (the ratio between the time spent exploring the object and the total exploration time), during the familiarization phase when the two objects were identical. Mice are the same as in panel A (n=11-18). Data are the mean ± s.e.m. No statistical difference was measured by t test.

5.3.5 Effects of the treatment with $PD1_{n\text{-}3DPA}ME$ on spontaneous seizures

$PD1_{n\text{-}3DPA}ME$ (n=9) or saline (n=12) were injected in mice for 4 days starting 1 h after SE induction. In order to check whether the treatment modified SE, its duration and spike number were measured. SE duration was not modified by the pro-resolving treatment (Saline, 7.7 ± 1.1 h; $PD1_{n\text{-}3DPA}ME$, 6.2 ± 1.0 h). The number of spikes calculated during 12 h of SE was similar between drug- and vehicle-injected mice (Saline, 51446 ± 9533; $PD1_{n\text{-}3DPA}ME$, 40671 ± 7249) confirming previous results (Section 5.3.3 and 5.3.4).

In order to assess the effect of the treatment on epilepsy development, rats were EEG recorded to detect the onset of the first two unprovoked spontaneous seizures. Representative EEG tracings of spontaneous seizures in the two experimental groups are depicted in Figure 5.9A. The onset of spontaneous seizures was not modified by $PD1_{n\text{-}3DPA}ME$ (Figure 5.10A; SE+Saline, 5.8 ± 0.6 days; SE+$PD1_{n\text{-}3DPA}ME$, 8.8 ± 2.8 days).

Then, the number of seizures occurring during 2 consecutive weeks after disease onset was calculated. The number of seizures per day and the total time spent in ictal activity were significantly reduced by 2-fold on average in mice treated with $PD1_{n\text{-}3DPA}ME$ (Figure 5.10B,C; number of seizures/day: SE+Saline, 1.6 ± 0.4; SE+$PD1_{n\text{-}3DPA}ME$, 0.7 ± 0.2; time in seizures: SE+Saline, 1030 ± 244 sec; SE+$PD1_{n\text{-}3DPA}ME$, 301 ± 82 sec). Finally, the average duration of seizures was significantly reduced in treated mice as compared to saline-injected mice (Figure 5.10D; SE+Saline, 40.2±3.6; SE+$PD1_{n\text{-}3DPA}ME$, 25.9±2.3 sec).

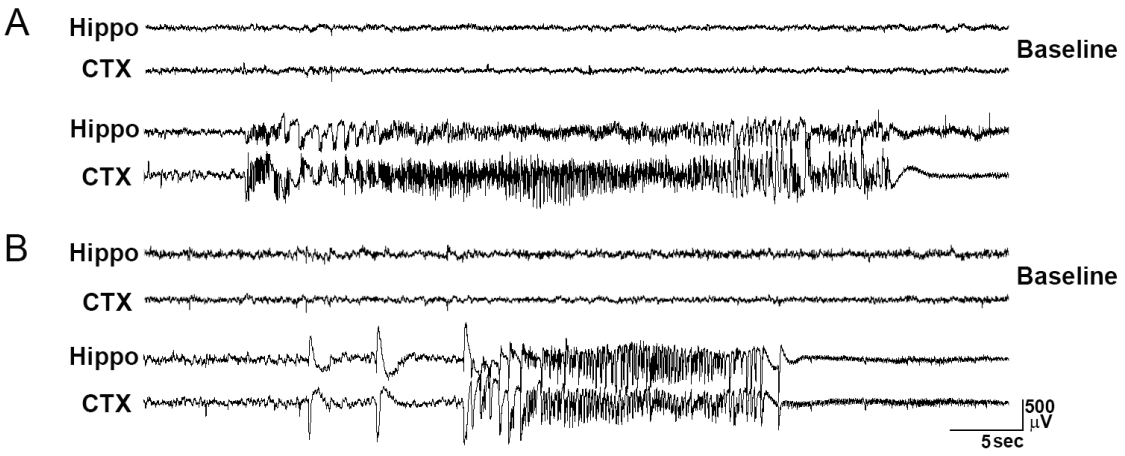


Figure 5.9. Representative EEG tracings depicting spontaneous seizures in mice treated with saline or PD1$_{n\text{-}3DPA}$ME. EEG tracings depicting baseline activity and spontaneous seizures as recorded in the right hippocampus (Hippo) and in the left cortex (Ctx) of mice treated with saline (panel A) or PD1$_{n\text{-}3DPA}$ME (panel B). Duration of spontaneous seizures is significantly reduced by the treatment as reported in Figure 5.10D.

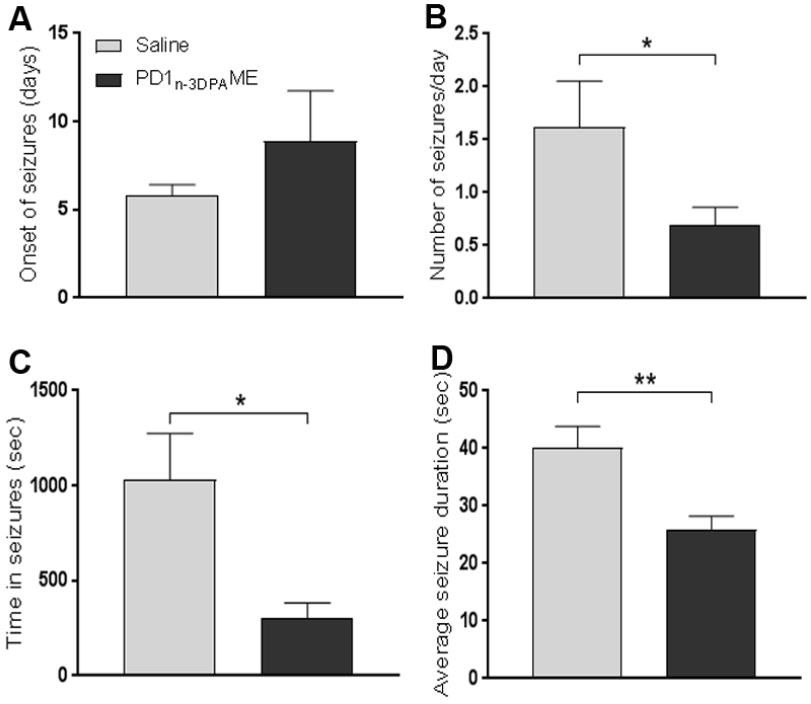


Figure 5.10. PD1$_{n\text{-}3DPA}$ME treatment reduced spontaneous seizure frequency and duration. Panel A depicts the onset of spontaneous seizures in mice treated with saline (n=12) or PD1$_{n\text{-}3DPA}$ME (n=9). Bargrams in panel B and C show the number of spontaneous seizures per day in the first two weeks after disease onset and cumulative time spent in seizures (sec) in saline vs treated mice. Panel D represents the average duration of spontaneous seizures (sec) in the two groups. Data are the mean ± s.e.m. ***** p<0.05; ****** p<0.01 vs Saline injected mice by one-tailed t test.

5.4 DISCUSSION

Treatment with both lipid (LXA_4 synthetic analogue) and peptide (Annexin A1 N-terminal derived peptide) ligands of ALXR was unable to resolve neuroinflammation. The presence of an up-regulation of ALXR, shown in Chapter 4, would in principle make the brain tissue more responsive to pro-resolving synthetic analogues. However, ALXR can also bind pro-inflammatory ligands, such as Aβ, serum amyloid protein A, IL-8 and CCL23 (Le et al., 2001) which could exacerbate the inflammatory response. There is evidence indicating that these molecules are upregulated in epileptogenic condition. Amyloid β-protein precursor (APP) is increased in temporal lobe epilepsy (Sheng et al., 1994) and electroconvulsive shock causes an increase in APP and Aβ expression in the hippocampus of mice (Jang et al., 2016). Previous studies have shown that APP synthesis and processing are induced by IL-1 which is increased after SE (Buxbaum et al., 1992). Consistently, neuronal expression of APP is associated with heightened IL-1 immunoreactivity in human temporal lobe epilepsy (Sheng et al., 1994). It is possible that SE induced release of IL-1 from glial cells leads to APP production in neurons and stimulation of ALXR during the initial phases of epileptogenesis. IL-8 concentration in serum and csf were increased rapidly after seizures in patients with refractory epilepsy, febrile SE, severe head injury and neonatal seizures induced by hypoxic-ischaemic encephalopathy and remained upregulated for 4 days thereafter (Billiau et al., 2007; Gallentine et al., 2017; Whalen et al., 2000; Youn et al., 2012). Finally, levels of CCL23 were upregulated in RE affected children (Owens et al., 2013).

We cannot exclude that the lack of resolution effect of the two treatments was due to failure to attain therapeutic levels due to the limited solubility of the two compounds in the small volume allowed by the icv injection (1-1.75 μl) and the limited agonist potency of BLM111 for ALXR (Jesmond Dalli's personal communication).

Lipidomic analysis showed a modified profile of lipid mediators during epileptogenesis as compared to controls. In particular, we detected a 20-fold up-regulation of DPA-derived

neuroprotectin PD1 which suggests a possible role of this molecule in the resolution mechanisms activated during epileptogenesis. The precursor of this novel protectin is n-3DPA which is present in human tissue and is relative abundant in human milk. It is an intermediate in the metabolic pathway of DHA (Figure 5.11) (Ferdinandusse et al., 2001; Hansen et al., 2017). DPA is an important component of our diet, along with EPA and DHA, and is enriched in fish, algae and seed oils. In recent years, numerous observational trials have demonstrated a clear link between DPA intake and improved health state, while multiple *in vitro* and *in vivo* studies have shown direct effects of DPA on inflammation, lipid metabolism and cognitive function (Byelashov et al., 2015).

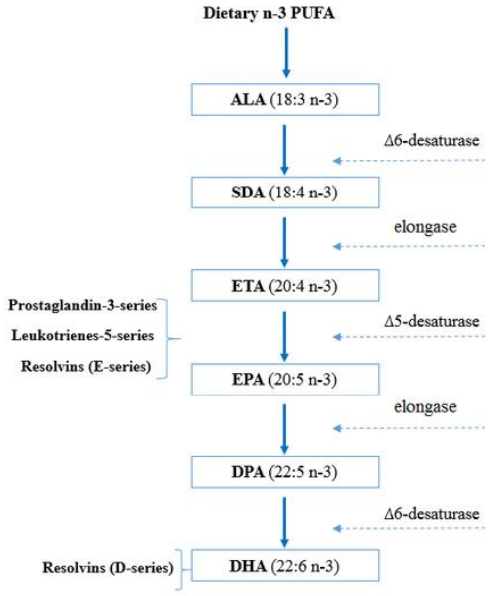


Figure 5.11. Synthetic pathways of long-chain PUFA and eicosanoids. *α -linolenic acid (ALA; 18:3 n-3) is an essential PUFA obtained from the diet, and after sequential desaturation and elongation steps, give rise to long chain, more unsaturated PUFA eicosapentaenoic acid (EPA; 20:5 n-3), docosahexaenoic acid (DHA; 22:6 n-3). Relevant intermediates in these pathways include SDA (stearidonic acid), ETA (eicosatetraenoic acid) and DPA (docosapentaenoic acid). EPA is a substrate for the synthesis of eicosanoid products such as prostaglandins (PG) and leukotrienes (LT).*

Because of the prominent increase in $PD1_{n\text{-}3DPA}$, we tested whether incrementing further its level using a synthetic stable analogue ($PD1_{n\text{-}3DPA}ME$) would counteract neuroinflammation. Our strategy was also based on the evidence that *in vivo* administration of this mediator reduced colitis through a mechanism partly linked to decreased inflammation (Gobbetti et al.,

2017). Moreover, administration of 10 ng of $PD1_{n\text{-}3DPA}ME$ per mouse significantly reduced neutrophil recruitment during peritonitis following zymosan A challenge, as determined by light microscopy and flow cytometry (Gobbetti et al., 2017). These actions were comparable to those displayed by the DHA-derived specialized pro-resolving mediator PD1 (Hansen et al., 2017). $PD1_{n\text{-}3DPA}$ differentiates from PD1 derived from DHA for an adjunctive double bond in the chemical structure and its effects has been less investigated. Treatment with PD1 in an experimental model of AD reduced microglia and astrocytes activation, decreased pro-inflammatory cytokines and inhibited caspase-3, thus favoring the expression of anti-apoptotic proteins (Zhao et al., 2011). PD1 also down-regulated pro-inflammatory signaling induced by cytokines and protected the mouse retina against oxidative stress (Mukherjee et al., 2004).

In this study, $PD1_{n\text{-}3DPA}ME$ induced a reduction of IL-1β expression during epileptogenesis when administered readily after SE, thus indicating it reduced neuroinflammation. The same treatment improved weight recovery in mice after SE and rescued non spatial memory deficits, thus indicating therapeutic effects on impaired cognition. Moreover, $PD1_{n\text{-}3DPA}ME$ reduced spontaneous seizure frequency and duration in the early stages of the disease, suggesting that implementing resolution could be a strategy to affect epileptogenesis. In order to test if this therapeutic effect is transient or persists during epilepsy development, further studies will evaluate the effect of the treatment on spontaneous seizures during the late stages of the disease.

Confirming our results, Musto and colleagues demonstrated that treatment with PD1 derived from DHA reduced seizure frequency 3 weeks after SE induced by pilocarpine in mice (Musto et al., 2015).

Since the molecular mechanism(s) of action of $PD1_{n\text{-}3DPA}$ are still unknown, further studies are required to get insights in the pathways activated by this specialized lipid mediator which mediate its anti-inflammatory and therapeutic effects (Hansen et al., 2017). There are few evidence indicating that PPARγ receptors are responsible for the effects of PD1 derived from DHA (Zhao et al., 2011). PPARγ can regulate lipid metabolism and inflammation: their activation

in macrophages promotes lipid clearance and inhibits the expression of inflammatory mediators, such as iNOS and IFNγ, and promotes the expression of anti-inflammatory molecules, such as IL-10 (Tontonoz and Spiegelman, 2008).

The data attained so far supported further investigations into the antiepileptogenic effects of $PD1_{n\text{-}3DPA}$. We will therefore determine if $PD1_{n\text{-}3DPA}ME$ treatment affects the progression of spontaneous seizures, as well as the neuropathology with a special focus on neurodegeneration and neurogenesis. These phenomena are associated with cognitive deficits in experimental models and appear to contribute to the establishment of the aberrant neuronal network underlying epileptogenesis.

CHAPTER 6 - EFFECTS OF A COMBINED ANTI-INFLAMMATORY TREATMENT DURING EPILEPTOGENESIS INDUCED BY ELECTRICAL SE

6.1 INTRODUCTION

Specific anti-inflammatory treatments have shown efficacy in reducing acute and chronic recurrent seizures in rodent models of epilepsy (Vezzani et al., 2011a). New evidence is arising demonstrating that such treatments may also mediate antiepileptogenic or disease-modifying effects (Terrone et al., 2016).

In particular, our laboratory has shown that the IL-1R1/TLR4 axis is activated by IL-1β and HMGB1 respectively during seizures and after epileptogenic injuries both in human epilepsy and in the corresponding experimental models (Vezzani et al., 2011b). Targeting the single components of this complex pathway drastically reduced induced or spontaneous seizures (Iori et al., 2013; Maroso et al., 2010). The IL-1β/IL-1R1 axis has been targeted also during epileptogenesis by using a combination of two anti-inflammatory drugs: anakinra, the recombinant IL-1R1 receptor antagonist, and VX-765, a selective caspase-1 inhibitor which prevents the biosynthesis of the mature and biologically active form of IL-1β. This combined treatment determined neuroprotection, but had no effect on epilepsy development (Noe et al., 2013) thus suggesting that the blockade of the IL-1β/IL-1R1 axis was not sufficient to arrest epileptogenesis. The same study provided additional evidence that blockade of the IL-1β/IL-1R1 axis did not affect TLR4 signaling activation which *per se* plays a role in epileptogenesis. In fact, mice deficient in TLR4 developed a milder form of epilepsy (Iori et al., 2013). These findings together raised the hypothesis that a combination of drugs targeting simultaneously both the IL-1R1 and TLR4 signalings was required to attain an efficient inhibition of epileptogenesis. Therefore, I applied a rational combination of anti-inflammatory drugs, namely anakinra and BoxA, a fragment of HMGB1 with antagonistic properties, using a treatment protocol previously shown to interfere with seizure mechanisms (Iori et al., 2013; Maroso et al., 2010; Vezzani et al., 2000). Ifenprodil was additionally used to block NR2B-NMDAR which represents one molecular target of IL-1β/IL-1R1 and HMGB1/TLR4 signaling mediating excitotoxicity (Balosso et al., 2008, 2014).

6.2 SPECIFIC MATERIALS AND METHODS

Electrical SE model

SE was induced by electrical stimulation of the CA3 region of ventral hippocampus (General materials and methods, Section 3.3.2 and 3.4.2). Rats exposed to electrical SE were randomized in treatment group (n=9) injected with a combination of anti-inflammatory drugs during epileptogenesis and placebo group (n=9) receiving the corresponding vehicles. The day before electrode implantation rats were anesthetized with 1.5% isofluorane and surgically prepared with jugular catheters (Cervo et al., 2013) for intravenous injection of drugs. This procedure lasted for about 30 min. Drugs or their vehicles were injected for one week starting 1 h after SE induction to encompass the epileptogenesis phase preceding disease onset, then treatment was stopped. Rats were video-EEG monitored continuously from the induction of SE until the first two spontaneous seizures occurred (i.e., epilepsy onset; General materials and methods, Section 3.4.2) and for 2 consecutive weeks at 2 and at 4 months post-SE to count spontaneous seizure number as the primary outcome measure (Figure 6.1, 6.2 and 6.4). A rational combination of anti-inflammatory drugs was used: anakinra, the human recombinant IL-1 receptor antagonist (Dinarello et al., 2012) (10 mg/200 µl/rat, intravenously, i.v., once/daily) and BoxA, a fragment of HMGB1 with antagonist activity (Andersson and Tracey, 2011) (100 µg/200 µl/rat, i.v., once/daily) together with ifenprodil, an antagonist of the NR2B-containing NMDA receptor (NMDAR) (Williams, 2001) (20 mg/kg, i.p, once/daily). A group of vehicle-injected rats were implanted with electrodes but were not exposed to SE and were used as SHAM control for time-matched blood HMGB1 measurements (n=9) (see data in Chapter 7). Rats were weighted before SE induction (baseline condition) and at 2 months and 4 months post-SE. Finally, rats were weighted before their sacrifice for histological brain analysis at 4.5 months post-SE (Figure 6.1 and 6.3).

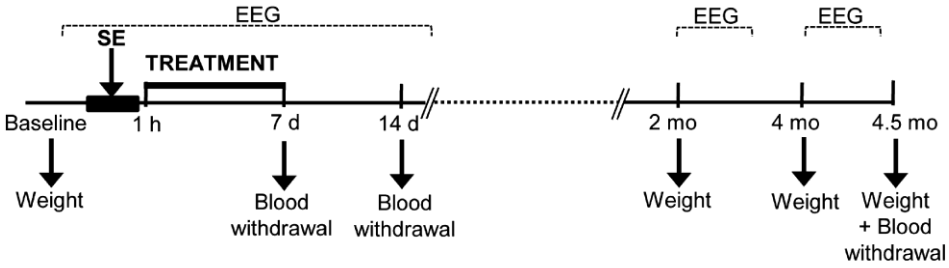


Figure 6.1. Experimental design in adult rats exposed to electrical SE. The time window of treatment administration post-SE and EEG recordings are reported, as well as the time points at which rats were weighted during the experiment. Time of blood withdrawal for HMGB1 measurement (Chapter 7) is indicated (data in Figure 7.4). Video-EEG recordings were done continuously from SE induction until the onset of the first two spontaneous seizures (epilepsy diagnosis), and for 2 consecutive weeks (24/7) at 2 and 4 months (mo) post-SE. One hour after SE induction, rats were randomized into treatment and vehicle group (n=9 rats each group). Treatment included anakinra+Box-A+ifenprodil (SE+DRUG). SHAM rats (n=9) were electrode-implanted but not stimulated, and received vehicles. Rats were sacrified at the end of the last EEG recording session for histological analysis of cell loss.

Analysis of SE, quantification of seizures and calculation of seizure progression index

No pharmacological intervention was done to stop SE after its onset since no mortality is observed in this model. SE severity was evaluated by EEG analysis by measuring its total duration and the number of spikes during the first 8 h using Clampfit 9.0 program (Axon Instruments, Union City, CA, U.S.A.). SE ended when spike frequency was lower than 1 Hz (1 spike/sec, see General materials and methods, Section 3.4.4).

The *progression index* was calculated for each rat as the ratio between the total number of seizures occurring between 4.0 and 4.5 months divided by the total number of seizures occuring from 2.0 to 2.5 months in the treatment and the vehicle-injected groups. The odds in each experimental group were calculated as the ratio between the number of seizures experienced by rats from 4.0 to 4.5 months and the total number of seizures from 2.0 to 2.5 months. The Odds Ratio was the ratio between the odds of treatment and vehicle groups.

Analysis of neurodegeneration

At the end of experiments, i.e. 4.5 months post-SE, rats were transcardially perfused and their brains were harvested (n=9 rats each group). Cell loss was measured as previously described in both hemispheres (Figure 6.5) (Filibian et al., 2012; Noé et al., 2013; Pascente et al., 2016). For a detailed protocol see General materials and methods, Section 3.6.2.

6.3 RESULTS

6.3.1 Effect of a combined anti-inflammatory treatment on SE and animal weight

Drugs or their vehicles were injected in rats for one week starting 1 h after SE induction. In order to check whether the drug combination modified SE, its duration and severity were measured. SE duration was not modified by the anti-inflammatory treatment (Figure 6.2A; SE+Vehicle, 8.7 ± 1.2 h; SE+Drug, 7.9 ± 0.4 h, n=9 each group). The number of spikes calculated during 8 h of SE, which is the average duration of SE in rats, was similar between drug- and vehicle-injected rats (Figure 6.2B; SE+Vehicle, 31426 ± 3826; SE+Drug, 38684 ± 2380). Figure 6.3 shows that rats exposed to SE and injected with vehicles significantly lost weight at 2 months as compared to their baseline and to sham rats. Rats treated with anti-inflammatory drugs did not lose weight during disease development thus indicating they were in a healthier state than their vehicle-injected SE-exposed controls.

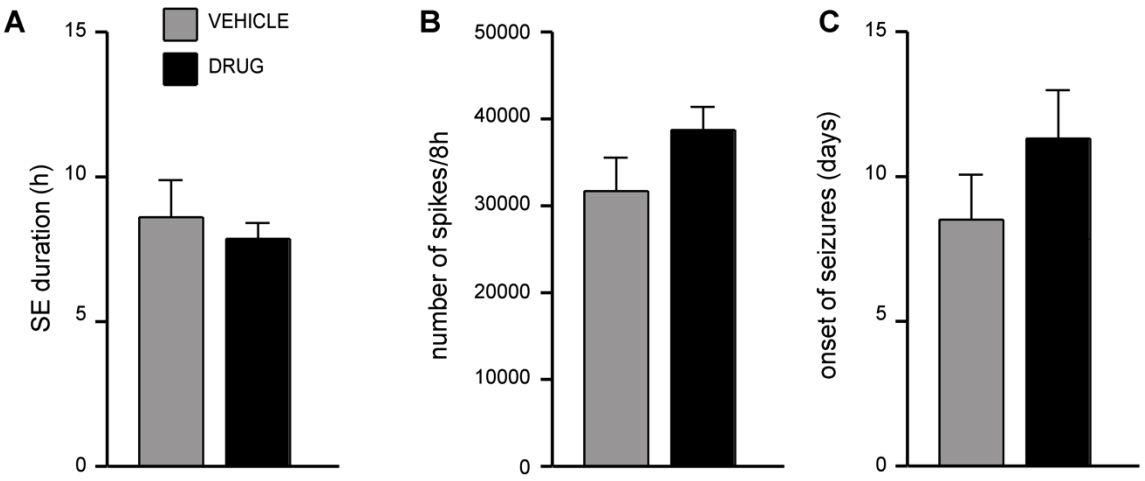


Figure 6.2. Anti-inflammatory drugs administered during epileptogenesis did not modify electrical SE and spontaneous seizure onset in rats. *Panel (A, B):* Quantitative measures of SE as assessed by EEG analysis, (A) SE duration and (B) number of spikes during 8 h of SE. *Panel (C):* Spontaneous seizure onset in vehicle- and drug-treated rats. Data are the mean ± s.e.m. (n=9 rats each group), no statistical difference was measured by Mann-Whitney test.

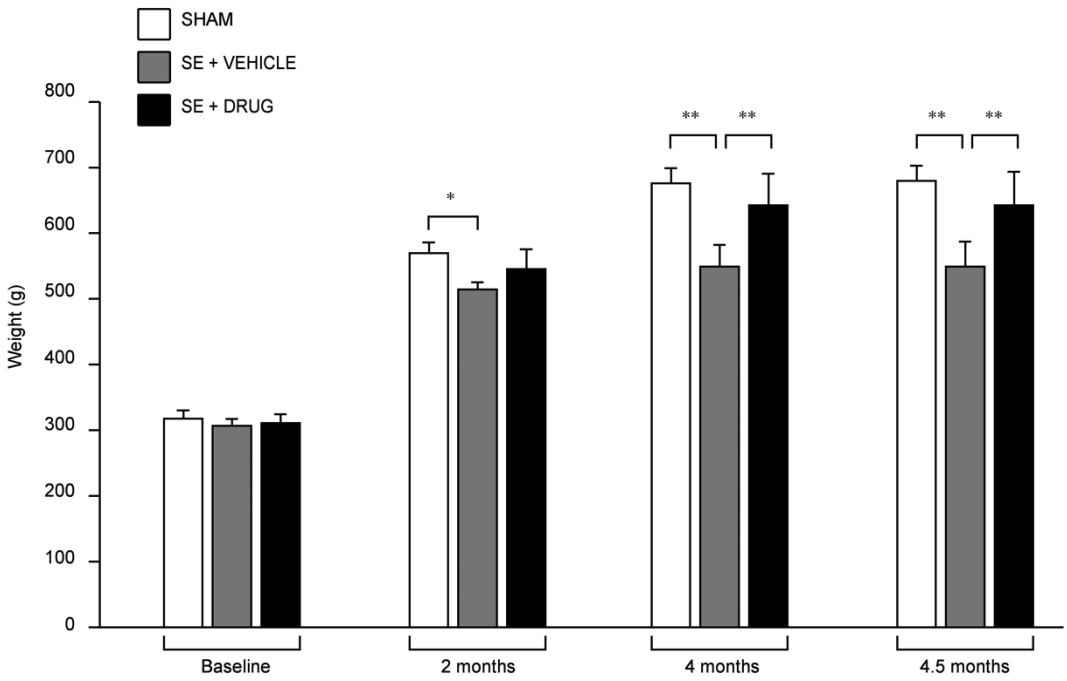


Figure 6.3. Anti-inflammatory drugs administered during epileptogenesis prevented weight loss in electrical SE-exposed rats. Bargrams report the weight of rats at baseline and post-SE during disease development in the vehicle and treatment groups as compared to SHAM rats. Data are the mean ± s.e.m. (n=9 rats each group). *p<0.05, **p<0.01 by Kruskal-Wallis test.

6.3.2 Effect of a combined anti-inflammatory treatment during epileptogenesis on spontaneous seizure onset and seizure progression

In order to assess the effect of the treatment on epilepsy development, rats were EEG recorded to detect the onset of the first two spontaneous seizures. The time of spontaneous seizure onset was not modified by the treatment (Figure 6.2C; SE+Vehicle, 8.4 ± 1.8 days; SE+Drug, 11.6 ± 1.7 days).

The number of seizures occurring during 2 consecutive weeks was reckoned at 2 and 4 months post-SE and the progression index was calculated (Table 6.1). In the 80% of vehicle-injected rats, seizure frequency was increased by 5-fold on average between 2 and 4 months after SE. The 45% of rats treated with the anti-inflammatory drug combination showed a 2-fold increase in seizure frequency, while the 33% of treated rats showed a 3-fold reduction in seizure frequency (Table 6.1).

Seven out of 9 rats in the vehicle group had a progression index higher than 1 indicating disease progression while in the remaining 2 rats there was no seizure progression. In the treatment group, 4 out of 9 rats showed a non-progressive disease and 1 rat did not experience any seizure (Table 6.1).

The progression index in rats exposed to SE and treated with anti-inflammatory drugs was significantly reduced as compared to SE-rats treated with vehicles (Figure 6.4A; SE+Vehicle, 4.6 ± 1.5; SE+Drug, 1.1 ± 0.4). The odds ratio (OR: 0.24; p=0.008) indicated that drug-treated rats at 2 months post-SE had a 76% reduced risk of developing seizures at 4 months vs vehicle-treated rats (Figure 6.4B).

Overall, these data show that the combined anti-inflammatory treatment had a disease-modifying effect by reducing the progression of the disease.

Table 6.1. *Number of spontaneous seizures and progression index in vehicle- and drug-treated rats exposed to electrical SE.*

Exp group	*Number of seizure at 2 months post-SE*	*Number of seizure at 4 months post-SE*	*Progression index*
Vehicle	9	41	4.56
Vehicle	3	8	2.67
Vehicle	6	8	1.33
Vehicle	3	20	6.67
Vehicle	0	2	n.a.
Vehicle	4	60	15.00
Vehicle	0	3	n.a.
Vehicle	7	3	0.43
Vehicle	2	1	0.50
Treatment	1	2	2.00
Treatment	4	10	2.50
Treatment	2	4	2.00
Treatment	29	75	2.59
Treatment	10	7	0.70
Treatment	11	3	0.27
Treatment	28	1	0.04
Treatment	15	0	0.00
Treatment	0	0	0.00

n.a.: not applicable refers to rats in which no seizures were detected at 2 months. For statistical analysis, these rats were attributed the average progression index measured in the corresponding vehicle group. Gray areas include rats which did not progress in each experimental group. All rats were used for statistical analysis.

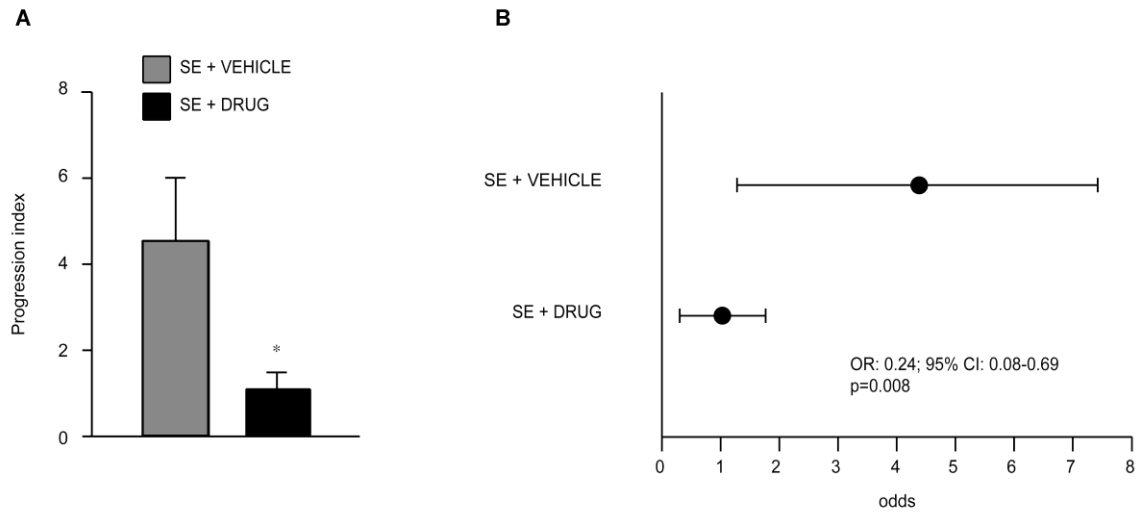


Figure 6.4. Anti-inflammatory drugs administered during epileptogenesis prevented disease progression in electrical SE-exposed rats. *Panel (A):* Progression index (i.e., the ratio between the number of seizures at 4 months and at 2 months post-SE) in vehicle- and drug-treated rats. A value=1 means lack of progression. Data are the mean ± s.e.m. (n=9 rats each group), *p<0.05 vs vehicle by Mann-Whitney test. Raw data in Table 6.1. *Panel (B)*: Odds ratio (OR: 0.24; p=0.008) indicates that drug-treated rats evaluated at 2 months have a 76% reduced risk of developing seizures at 4 months vs vehicle-treated rats. Treatment included anakinra+BoxA+ifenprodil (SE+DRUG) (detailed protocol in Specific materials and methods, Section 6.2).

6.3.3 Effect of a combined anti-inflammatory treatment on neurodegeneration

Neurodegeneration was assessed by Nissl staining in drug- and vehicle-injected rats in the chronic epileptic phase at the end of EEG recording. In accordance with previous evidence in the same rat model of electrical SE (Noe et al., 2013; Pauletti et al., 2017), there was a significant neuronal cell loss in hippocampus and parahippocampal areas (entorhinal and frontoparietal cortices) as compared to sham rats (Figure 6.5). The drug combination did not provide neuroprotection since it did not modify the number of CA1 and CA3 pyramidal neurons, hilar interneurons, or neurons in entorhinal and frontoparietal cortices as compared to vehicle-injected rats (Figure 6.5).

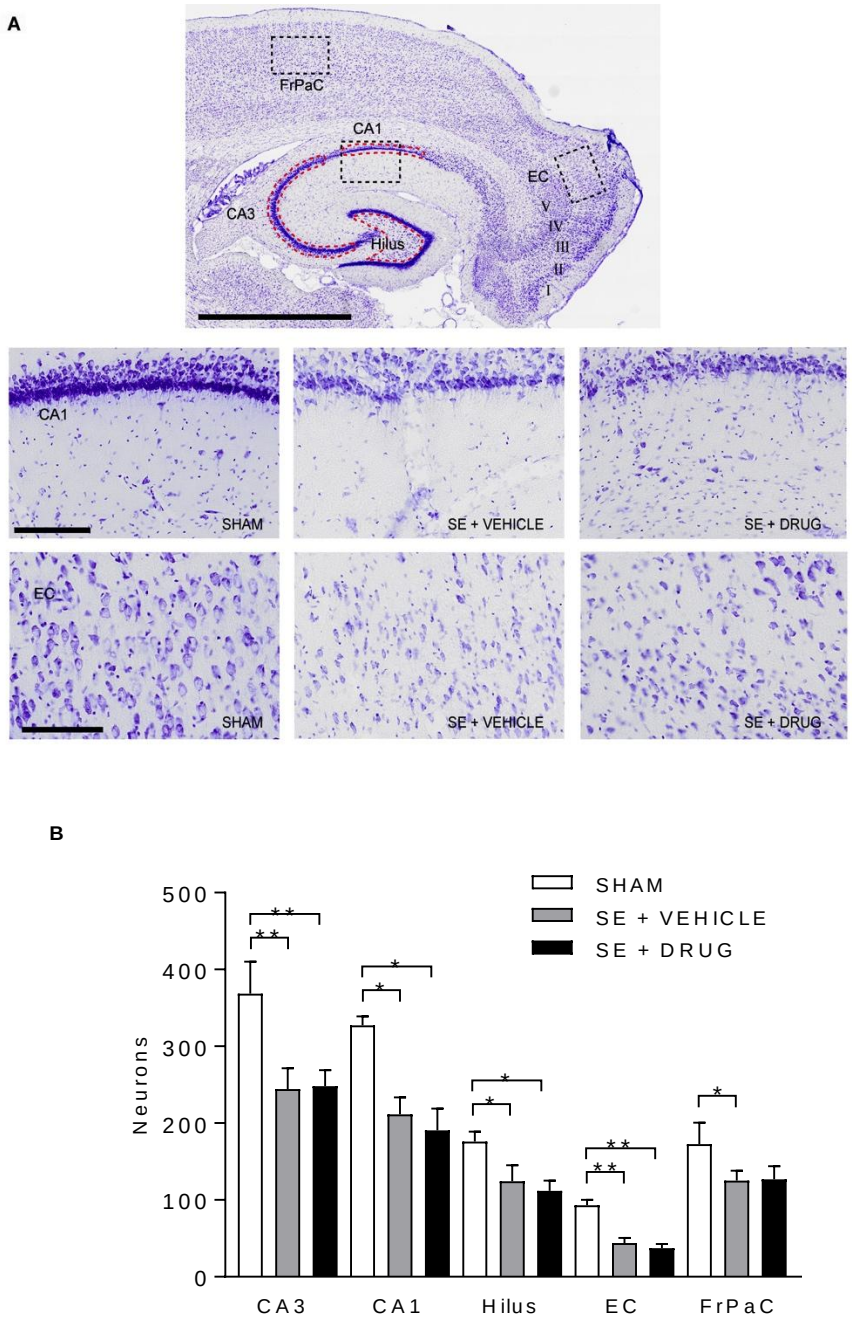


Figure 6.5. Anti-inflammatory drugs administered during epileptogenesis did not modify neurodegeneration in electrical SE-exposed rats. *Panel (A)* shows representative photomicrographs of Nissl-stained hippocampal sections. *First row*, boxed and dotted areas in the hippocampal slice represent where cell quantification was done. *Panel (B)* shows the relative quantification of neuronal cell loss in rats exposed to electrical SE and treated with vehicle or anti-inflammatory drugs for 1 week (n=9 each group). Rats were sacrified 4.5 months post-SE (protocol in Figure 6.1). Data are expressed as absolute number of neurons (mean ± s.e.m. ; n=9 rats each group). *p<0.05, **p<0.01 by Kruskal-Wallis test. *Scale bar*: 250 µm; 50µm. EC= entorhinal cortex; FrPaC= frontoparietal cortex.

6.4 DISCUSSION

The anti-inflammatory drug combination administred during epileptogenesis did not modify the acute epileptogenic injury, namely SE, thus its therapeutic effects were not due to modifications of the initial insult. The anti-inflammatory drug combination showed a disease-modifying effect since it reduced the progression of seizures during the disease development and improved the health state of the animals, which is otherwise compromised by SE, as shown by the prevention of body weight loss compared to vehicle-injected SE rats. In rats treated with the anti-inflammatory drug combination, although the progression index is significantly reduced, the number of seizures at 2 months post-SE trends to be higher than in vehicle-treated rats. It is possible that this apparent initial worsening of seizures as opposed to the reduced seizures at the late stage of epilepsy is due to the interference with protective inflammatory responses activated in the first hours following SE (Serhan et al., 2005). In support of this data interpretation is the evidence that our treatment protocol did not afford neuroprotection whereas anti-inflammatory drugs admnistration when started 3 h after SE was indeed neuroprotective (Noé et al., 2013). A longer treatment schedule might be also required for attaining neuroprotection with the present drug combination. The lack of neuroprotective effect with the present drug combination may also be due to additional differences in the treatment design in the two studies. (1) Different combinations of drugs were used, namely VX-765 and anakinra (Noé et al., 2013) vs anakinra, ifenprodil and BoxA. (2) Administration protocols were different since anakinra was continuously infused subcutaneously by osmotic minipumps in the previous study (Noé et al., 2013) while in the present study anakinra was administered once daily by intravenous injection.

This study demonstrates that a combined anti-inflammatory treatment transiently administered after the epileptogenic insult, although not preventing epilepsy onset, significantly arrested disease progression. In accordance with our data, there is evidence that: (1) an anti-inflammatory drug combinations using anakinra and a COX-2 inhibitor, but not each

drug given alone, reduced chronic spontaneous seizures (Table 1.4.3) (Kwon et al., 2013); (2) an epigenetic approach using miRNA146a which reduces IRAK1/2 and TRAF6 therefore inhibiting both the IL-1R1 and TLR4 signaling (Quinn and O'Neill, 2011), or a combination of VX-765 and a TLR4 antagonist, prevented disease progression and reduced up to 90% chronic seizure recurrence (Iori et al., 2017b).

Altogether, these data show that combined anti-inflammatory treatments is a promising strategy to effectively interfere with epileptogenesis.

CHAPTER 7 - MOLECULAR ISOFORMS OF HMGB1 AS NOVEL BIOMARKERS FOR EPILEPTOGENESIS

7.1 INTRODUCTION

An unmet clinical need in epilepsy is the lack of non invasive biomarkers of epileptogenesis which could be easily measureable, such as soluble molecules detectable in the blood, and possibly reflecting epileptogenesis mechanisms taking place in the brain. Neuroinflammation is induced and plays a pivotal role in epileptogenesis (Vezzani et al., 2013). More specifically, IL-1R1/TLR4 axis is activated during seizures and epileptogenesis and its pharmacological targeting mediates disease modifying effects, as previously described and discussed in Chapter 6. The endogenous danger signal, HMGB1, promotes neuroinflammation by activating TLR4. HMGB1 is increased in neurons and glia in human drug-resistant epileptic foci (Maroso et al., 2010; Zurolo et al., 2011) and in the corresponding animal models (Maroso et al., 2010). The disulfide isoform of HMGB1 which is generated by oxidative stress promotes seizures and cell loss (Balosso et al., 2014; Iori et al., 2013; Maroso et al., 2010) and contributes to cognitive deficits (Mazarati et al, 2013). Moreover, mice lacking TLR4 are intrinsically less susceptible to seizures and less prone to develop epilepsy (Iori et al., 2013; Maroso et al., 2010).

Based on this evidence, HMGB1, which is readily measurable in blood, might be a valuable biomarker of neuroinflammation and as such it may predict the therapeutic response to anti-inflammatory drugs. Moreover, since this molecule is implicated in seizure mechanisms it might predict the development of epilepsy and the disease course.

The first objective of this study was therefore to establish whether a relationship exists between brain and blood levels of HMGB1 during the acute injury (SE) and early epileptogenesis. First, HMGB1 translocation from the nucleus to the cytoplasm was studied during epileptogenesis since this step is required for its cellular release, hence the consequent biological effects (Lu et al., 2014), and its potential brain-to-blood transfer. To probe and validate the potential biomarker role of HMGB1 for epileptogenesis, two different rat models of epileptogenesis were used. In the first model, 100% of animals developed epilepsy after SE evoked by electrical stimulation of hippocampus in adult rats (Lothman et al., 1989). In the

second model, about 70% of animals developed epilepsy after SE induced by lithium+pilocarpine in pre-adolescent PN21 rats. The remaining 30% of rats did not develop seizures, although all rats were exposed to a similar primary insult. Finally, to test HMGB1 as a biomarker of treatment response, its blood levels were measured in rats treated with the combined anti-inflammatory drugs that determined disease-modifying effects (Chapter 6).

7.2 SPECIFIC MATERIALS AND METHODS

Eletrical SE model

SE was induced by electrical stimulation of the CA3 region of ventral hippocampus (see General materials and methods, Section 3.3.2 and 3.4.2) followed by onset of spontaneous seizures at 8.4 ± 1.8 days post-SE. Three distinct cohorts of rats underwent different procedure for sample collection depending on the analysis to be performed.

The first set of rats was transcardially perfused at 3 h, 6 h and 4 days (n=5 rats in each group) post-SE and used for HMGB1 immunohistochemical analysis (Figure 7.2).

The second set of rats was decapitated at 3 h, 6 h and 4 days (n=5 rats in each group) after rapid PBS perfusion and used for measurement of HMGB1 and its isoforms in the hippocampi and in the venous blood collected from the heart atrium in each rat (before transcardial perfusion; Figure 7.3).

The third set of rats was used for the pharmacological experiment (data in Chapter 6 and Figure 7.4). Electrically-stimulated animals developing SE were randomly divided into 2 experimental groups (n=9/each group) and treated with vehicle (SE+Vehicle) or a combination of anti-inflammatory drugs (SE+DRUG; as previously reported in Chapter 6). In these rats, blood was drawn longitudinally at three time points post-SE (as indicated in Figure 6.1) representative of disease development: 7 days (*prodromal phase,* preceding spontaneous seizure onset), 14 days (encompassing the *time of disease onset*) and 4.5 months (*chronic epilepsy phase*) (General materials and methods, Section 3.4.2).

For the three different sets of experiments, sham-operated animals were implanted with electrodes but not electrically stimulated (SHAM, n=5-9) and were used as vehicle-injected controls for biochemical or histological analysis.

Lithium+Pilocarpine SE model

SE was evoked by systemic lithium+pilocarpine administration in PN21 rats (General materials and methods, Section 3.3.3 and 3.4.3). Around 70% of rats develop epilepsy in this model after an average time of 70 days (Marcon et al., 2009; Pascente et al., 2016). Accordingly, 12 out of 17 rats developed spontaneous seizures after being exposed to SE of similar severity and duration. Retrospective score analysis was done after rats were identified as epileptic or non-epileptic: Score 1 (min), non-epileptic 5.0±2.8, n=5; epileptic, 3.9±0.6, n=12; Score 2 (min), non-epileptic 10.8 ± 2.5; epileptic, 10.2 ± 1.4 (General materials and methods, Section 3.4.3). The onset of SE was determined by the time of appearance of the second stage 5 seizure and was similar in non-epileptic (12.2 ± 2.3 h, n=5) and epileptic rats (11.7 ± 1.5 h, n=12): after this time stage 5 seizures similarly recurred in both groups for the 6 h observation period (General materials and methods, Section 3.4.3 and 3.4.4).

In the chronic epileptic phase, we recorded rats for 2 weeks since this is an adequate time interval for detecting seizures based on their frequency, that is one seizure every 2-3 days (General materials and methods, Section 3.4.3 and 3.4.4). Epileptic rats experienced 3.7 ± 0.5 clinical seizures while no seizures were observed in non-epileptic rats. Although we cannot exclude that seizures might have occurred at a later time interval in *non-epileptic* rats, this appears unlikely based on literature evidence related to this model (Kubova et al., 2004; Sankar et al., 2000).

Epileptic rats also displayed a significant reduction in their threshold to after-discharge induction (116 ± 38 μA, n=12; $p<0.05$) as compared to sham controls (220 ± 40 μA, n=7); after-discharge threshold in non-epileptic rats without spontaneous seizures was similar to controls

(190 ± 40 μA, n=5) (Figure 7.1; General materials and methods, Section 3.4.3). This data show that hippocampal excitability was chronically enhanced only in rats that developed spontaneous seizures.

HMGB1 blood measurement was done in randomly selected epileptic rats (4.0 ± 1.3 seizures/2 weeks) and non-epileptic rats (n=5 each group) and compared with 7 vehicle-treated SHAM controls. In this rats, blood was drawn longitudinally post-SE at 23 days (*prodromal phase*), 73 days (encompassing the *time of disease onset in 70% of rats*) and 7.5 months (*chronic epilepsy phase*) (Figure 7.1; General materials and methods, Section 3.4.3). SHAM animals were vehicle-injected (not exposed to SE) and used as controls for blood HMGB1 measurements and histological brain analysis (n=5).

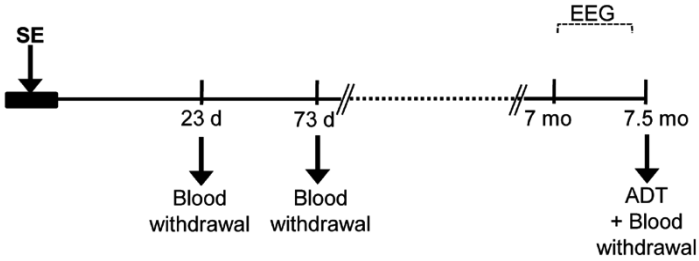


Figure 7.1. Experimental design in PN21 rats exposed to SE induced by lithium+pilocarpine. The scheme depicts the time points of blood withdrawal, EEG recording (2 weeks, 24/7) and afterdischarge threshold (ADT) in PN21 rats exposed to SE induced by lithium+pilocarpine. Data related to this protocol are reported in Figure 7.6 and Table 7.2.

Immunohistochemistry

HMGB1 immunohistochemistry was done in order to assess its nuclear vs cytoplasmatic cellular localization in the hippocampus of rats exposed to electrical-SE and perfused after 3 h, 6 h and 4 days compared with time-matched sham controls (Figure 7.2; General materials and methods, Section 3.6.1). Co-localization of HMGB1 signal with GFAP, OX-42, and EBA, a marker of endothelial cells of the BBB was done to define the cell phenotype (see General materials and methods, Section 3.6.1). HMGB1 and GFAP immunohistochemistry was also performed at 7.5 months post-SE induced by lithium+pilocarpine in PN21 rats (Figure 7.6).

Blood collection in rat epileptogenesis models

In the cross-sectional studies done in electrical SE rats, blood was collected by the heart atrium (before transcardial perfusion) at 3 h, 6 h and 4 days after SE onset; in longitudinal studies blood was drawn at representative times of disease development in each model: in electrical SE rats, blood was collected by the jugular catheter in lightly restrained animals (see Section 6.2); in PN21 rats, blood was collected longitudinally from the tail vein in animals under light isofluorane anaesthesia placed on a warming pad to avoid hypothermia.

Blood was drawn (~ 500 µl) using a butterfly (needle 21G) and collected in microtainer tubes. Plasma was isolated according to standard procedures, aliquoted and stored at -80°C until assayed. This procedure was carried out within 4 h from blood withdrawal. It has been described that total HMGB1 levels do not change when blood samples are processed and stored within 4 h (unpublished personal communications and outcome from scientific meeting in 2010, 'HMGB1 and tissue damage' Helsinki 2010). Moreover, Daniel Antoine's lab has conducted studies which support this observation confirming that HMGB1 redox modifications remain consistent when samples are also processed within 4 h (unpublished).

ELISA and liquid chromatography-tandem mass spectrometry analysis (LC/MS-MS)

Dr. Daniel Antoine (Centre for Drug Safety Science, University of Liverpool, Liverpool, UK) performed ELISA of total HMGB1 in blood and LC/MS-MS of HMGB1 isoforms in both hippocampus and blood of rats that were sacrificed 3 h, 6 h and 4 days after electrical SE (Figure 7.3). HMGB1 was also measured in blood in longitudinal studies in both electrical and lithium+pilocarpine SE models (Figure 7.4 and 7.6).

The proteolytic digestions of HMGB1 required for LC/MS-MS analysis generates peptide fragments that either contain the acetylation sites or the redox sensitive sequence (General materials and methods, Section 3.7.5). HMGB1 isoforms have been determined in blood serum

or plasma by a patented (USA US8748109 B2, Europe EP2449378, Japan 5721707) absolute quantification method (Antoine et al., 2009, 2012), which has been extensively validated to bioanalytical guidelines set out by the SAFE-T IMI funded biomarker consortium (http://www.imi-safe-t.eu/).

Since a method for absolute quantification of HMGB1 isoforms has not been validated for brain tissue as yet, HMGB1 isoforms in hippocampus are expressed as fold-change of respective basal values.

The results shown in Figures 7.3, 7.4 and 7.6 depict acetylated HMGB1 separately from the reduced and disufide isoforms. Since reduced and disulfide HMGB1 are mutually exclusive isoforms, their amounts account for total HMGB1 levels.

7.3 RESULTS

7.3.1 Brain expression of HMGB1 in the electrical SE model

In control tissue, HMGB1 was expressed in nuclei of neurons and glia (Figure 7.2A). From 3 h until 4 days post-SE, a time window preceding the onset of epilepsy in this model- as shown by the lack of EEG and motor seizures in rats under continuous video-EEG recording- HMGB1 staining showed a progressive increase in GFAP-positive astrocytes (Figure 7.2A,B). Notably, in all SE-exposed rats, HMGB1 staining was observed in the cytoplasm of glial cells, including activated OX-42-positive microglia, and in EBA-positive endothelial cells of the BBB (Figure 7.2A), and was detectable in the cytoplasm of neurons in 30% of animals. These data show that HMGB1 translocates from nucleus-to-cytoplasm before the onset of spontaneous seizures, therefore demonstrating that its brain changes are not a mere consequence of seizure activity.

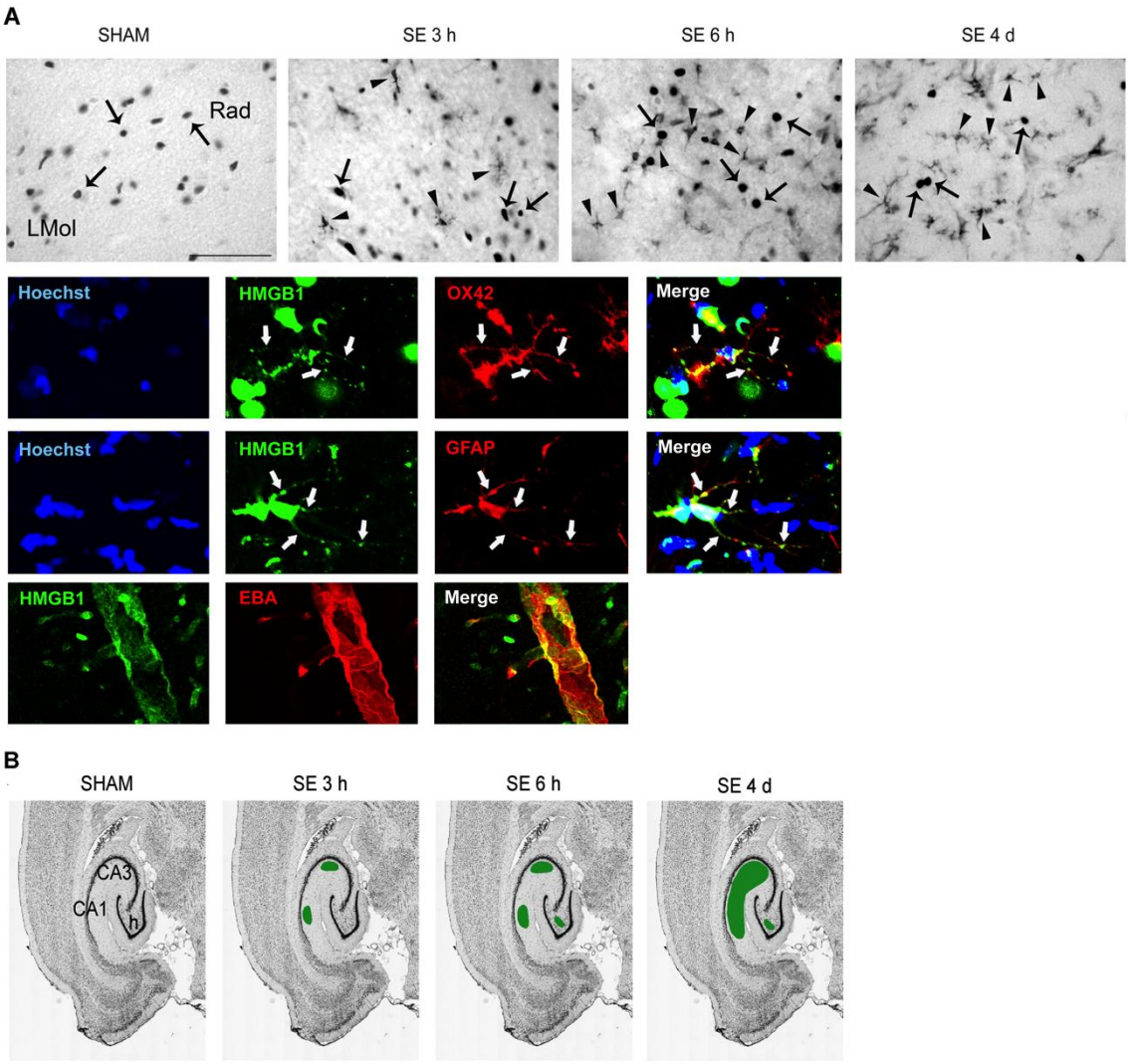


Figure 7.2. Brain HMGB1 expression during epileptogenesis evoked by electrical SE in adult rats. *Panel (A)*: Representative photomicrographs of hippocampi from control rats (SHAM) or rats at 3 h, 6 h and 4 days post-SE (n=5 each group). First row shows HMGB1 immunoreactivity in cell nuclei (arrows) or in cytoplasm of glial cells (arrowheads). Second row shows localization of HMGB1 signal (green) in OX-42-positive microglia (red), GFAP-positive astrocytes (red) and EBA-positive endothelial cells (red); co-localization signal is depicted in yellow (merge). White arrows depict cytoplasmic staining. Hoechst-positive nuclei are shown in blue. Rad: stratum radiatum; Lmol: stratum lacunosum moleculare. Scale bar: first row 25 µm; second row 12 µm. *Panel (B)*: Schematic representation of the progressive increase in cytoplasmatic HMGB1 staining in astrocytes during epileptogenesis. CA1, CA3 pyramidal cell layer; h: hilus.

7.3.2 Blood levels of HMBG1 in the electrical SE model

We examined whether an association exists between brain and blood HMGB1 isoforms in the same animals (Figure 7.3) In brain lysate from hippocampal tissue, the acetylated isoform (indicator of active release) progressively increased between 3 h and 4 days post-SE, i.e. during a time frame preceding the onset of spontaneous seizures. The redox state of HMGB1 also changed during epileptogenesis: at 3 h post-SE, only reduced HMGB1 was formed (3-fold increase over sham value) while between 6 h and 4 days, disulfide HMGB1 was also generated together with reduced HMGB1. In the blood of the same animals, the acetylated and disulfide isoforms increased with a delay of at least 6 h when compared to brain, and before spontaneous seizure onset. The reduced isoform increased in blood concomitantly with brain changes and, as in brain, anticipated the generation of disulfide HMGB1 which appeared in blood at 4 days. Within the first 6 h from SE induction, circulating HMGB1 was non-acetylated, likely reflecting its passive release from damaged cells, while at 4 days the majority of HMGB1 was acetylated, indicating active cellular release (Figure 7.3). It has previously been shown that the active cellular release of HMGB1 is through a Ca^{2+}-dependent secretory lysosome mechanism which, in turn, depends on HMGB1 acetylation (Antoine et al., 2009; Gardella et al., 2002).

These blood changes may reflect csf-to-blood transport of HMGB1 which is initially (within the first 6 h) passively released from damaged neurons (Dingledine et al., 2014; Maroso et al., 2010), and possibly also from BBB endothelium (Gorter et al., 2015; Lotowska et al., 2008). In particular, at 3 h post-SE, non-acetylated and reduced HMGB1 in blood overshot the brain changes by about 2-fold. Endothelial cells of BBB can contribute to this blood increase since they showed increased expression of HMGB1 during epileptogenesis (Figure 7.2A), and they can be damaged by SE (Parfenova et al., 2012).

The progressive active release of acetylated HMGB1 may originate mainly from astrocytes, as suggested by both the prominent astrocytic HMGB1 expression and its cytoplasmic translocation by immunohistochemistry (Figure 7.2A,B).

Notably, the brain changes in both acetylated and disulfide isoforms preceded the respective blood increases supporting a csf-to-blood transport.

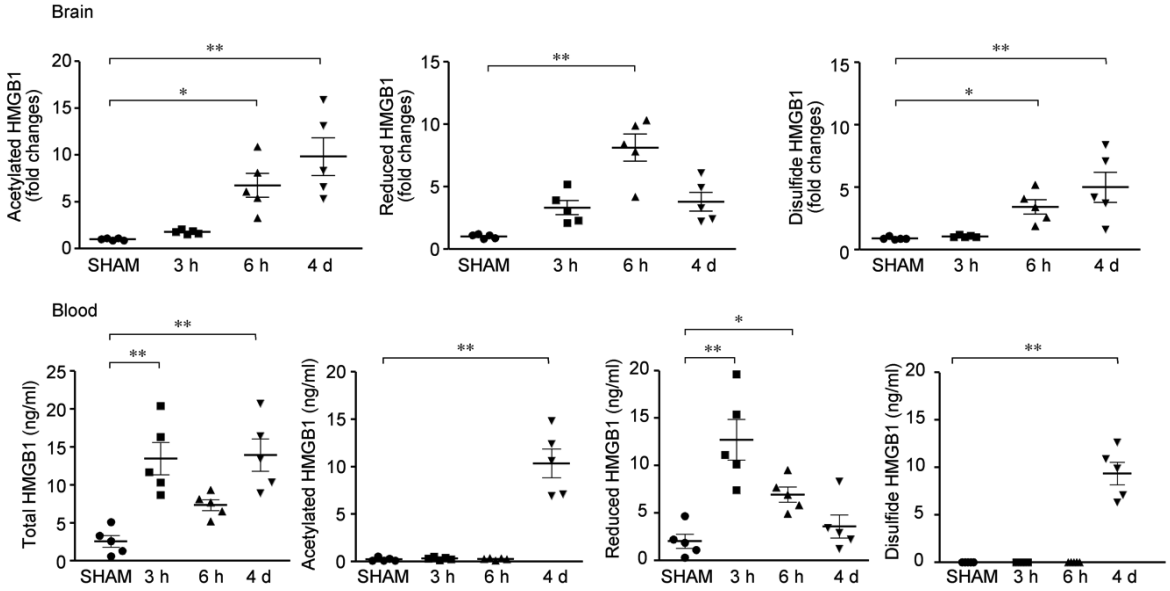


Figure 7.3. Brain and blood HMGB1 expression levels during epileptogenesis evoked by electrical SE in adult rats. *Upper row,* levels of HMGB1 isoforms in brain tissue (hippocampus) and corresponding blood of rats (*lower row*) during epileptogenesis. Individual rat values are reported as dot plots (n=5 each group). *p<0.05, **p<0.01 by Kruskal-Wallis test.

7.3.3 HMGB1 as a biomarker of epileptogenesis and treatment response in the electrical SE model

To investigate the value of blood HMGB1 in predicting epilepsy development, we initially used the electrically-induced SE model (also used in Figure 7.2 and 7.3) where all rats develop epilepsy. This model allowed to test whether blood HMGB1 levels change before the onset of spontaneous seizures, thus independently of ongoing seizure activity.

There was a progressive increase of both total HMGB1 and its acetylated and disulfide isoforms (the latter being undetectable in SHAM rats) in blood during disease development in vehicle-injected SE-exposed rats. Notably, these changes were detected before the onset of

spontaneous seizures (i.e. disease onset) as assessed in continuously video-EEG monitored rats, and persisted until the chronic phase of spontaneous seizures (Figure 7.4). Before disease onset, acetylated HMGB1 was the predominant isoform (70% of total HMGB1) reflecting active release. This isoform plateaued early after disease onset (50% of total HMGB1) and increased again in the chronic disease phase (4.5 months; 80% of total HMGB1). Reduced HMGB1 stably increased around 4-fold throughout the observation period (Figure 7.4). The inactive sulfonyl HMGB1 was not detected at any time point.

The anti-inflammatory drug combination significantly attenuated the increase in total HMGB1 and its isoforms during the prodromal phase, and abrogated blood HMGB1 changes after disease onset (Figure 7.4). Notably, the drug combination prevented the increase in seizure frequency determined by EEG analysis between 2 and 4.5 months post-SE in vehicle-injected rats (Chapter 6; Table 6.1 and Figure 6.4), thus prevented disease progression. There was no correlation between the number of chronic seizures in the 2-week recording period preceding blood withdrawal (4.5 months post-SE) and corresponding level of total blood HMGB1 (Figure 7.5).

Receiver Operating Condition (ROC) analysis (Table 7.1) showed that both total HMGB1 and its isoforms reliably differentiated between SHAM control and epileptic rats (AUC=1) in the chronic phase of spontaneous seizures supporting their value as diagnostic biomarkers of active epilepsy. Additionally, total HMGB1 and its isoforms also predicted with high fidelity (AUC=0.9-1 range) the therapeutic response to anti-inflammatory drugs before disease onset and at epilepsy diagnosis.

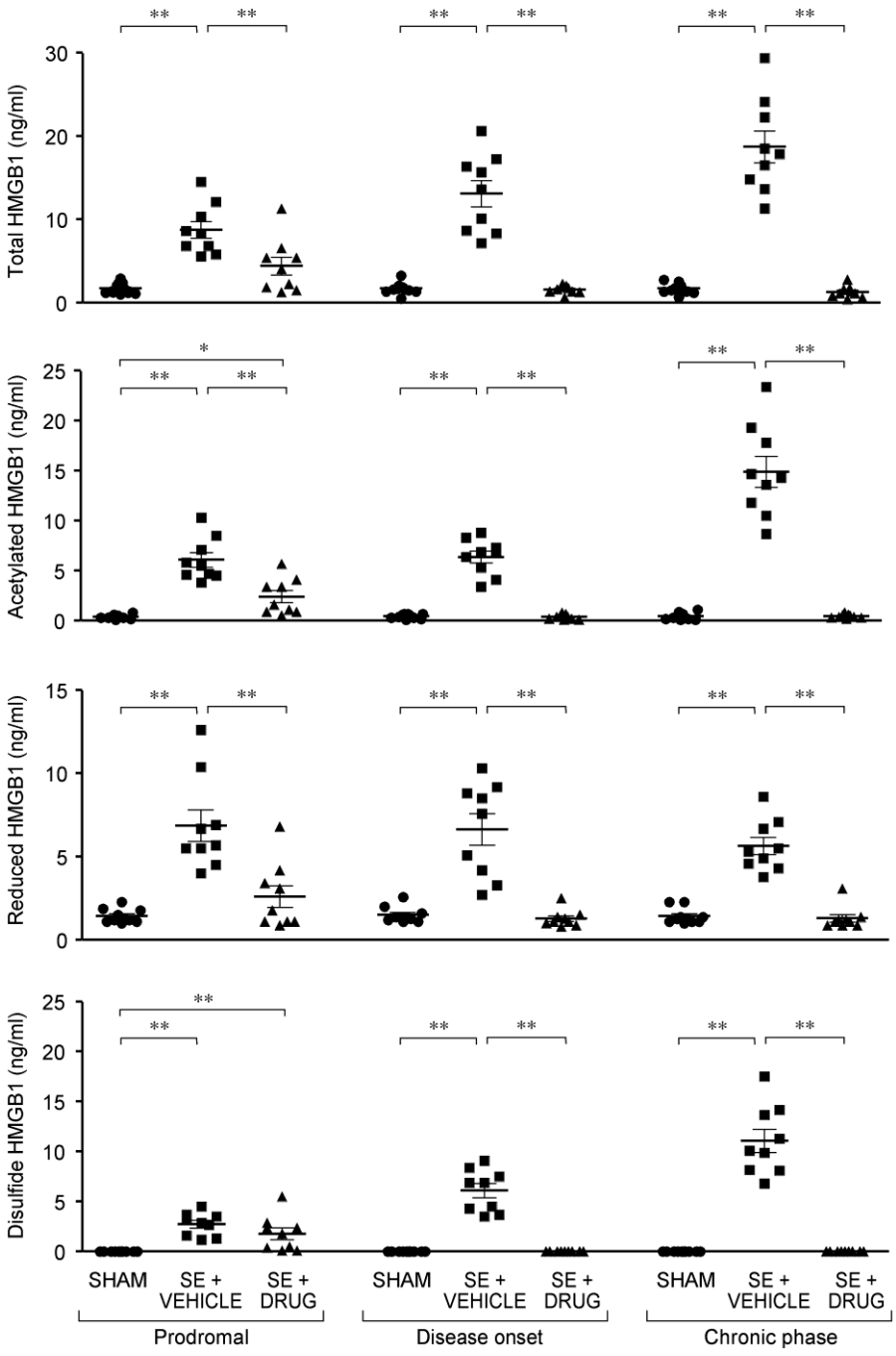


Figure 7.4. Blood HMGB1 levels increased before epilepsy onset in electrical SE-exposed rats, and predicted therapeutic response to anti-inflammatory drugs. Longitudinal analysis of total HMGB1 and levels of acetylated, reduced and disulfide isoforms in blood serum at representative time points of disease development in SE-exposed rats receiving treatment (DRUG) or corresponding VEHICLE. Blood was drawn at 7 days (epileptogenic phase prodromal to epilepsy onset), 14 days (encompassing the time of disease onset) and 4.5 months (chronic epilepsy phase). Treatment included anakinra+BoxA+ifenprodil (SE+DRUG). Rats are the same as in Chapter 6 (Figure 6.3, 6.4, 6.5 and Table 6.1). Individual rat values are reported as dot plots (n=9 rats each group). *p<0.05, **p<0.01 by Kruskal-Wallis test. The acetylated isoform level in the chronic phase (SE+VEHICLE) was significantly different from corresponding level at disease onset and prodromal phases (p<0.01 by repeated measures one-

way ANOVA). The disulfide isoform (SE+VEHICLE) level at disease onset and in the chronic phase was significantly different from corresponding level in prodromal phase ($p<0.05$ and $p<0.01$, respectively); the disulfide isoform level in the chronic phase was significantly different from corresponding level at disease onset ($p<0.05$ by repeated measures one-way ANOVA).

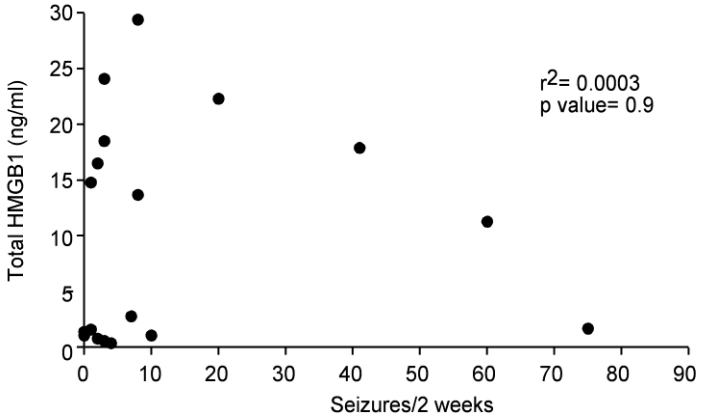


Figure 7.5. Blood HMGB1 levels in the chronic phase did not correlate with seizure frequency. Lack of correlation between seizure counts in rats with chronic epilepsy as assessed during 2-week video-EEG recording 4.0-4.5 months post-SE and the relative HMGB1 blood levels collected 4.5 months post-SE (total n=18: SE+VEHICLE, n=9 and SE+DRUG, n=9). These are the same rats as in Figure 7.4.

Table 7.1. *Receiver Operating Condition (ROC) analysis in electrical SE-exposed rats injected with vehicle or anti-inflammatory drugs.*

	VEHICLE vs TREATMENT *Prodromal phase*				VEHICLE vs TREATMENT *Disease onset*				Sham vs Epileptic *Chronic phase*			
	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)
Total HMGB1	0.9	0.0052	0.5 (0.2-0.8)	1.1	1	0.0005	1 (0.6-1)	10.5	1	0.0002	1 (0.7-1)	3
Ac-HMGB1	0.9	0.0028	0.9 (0.5-1)	3.9	1	0.0005	1 (0.6-1)	5.6	1	0.0002	1 (0.7-1)	1.5
Reduced HMGB1	0.9	0.0028	0.9 (0.5-1)	3.9	1	0.0005	1 (0.6-1)	3.7	1	0.0002	1 (0.7-1)	2.2
Disulfide HMGB1	0.8	0.0833	0.5 (0.2-0.8)	1.1	1	0.0005	1 (0.6-1)	3.7	1	0.0002	1 (0.7-1)	3

ROC analysis shows that blood HMGB1 level discriminates between control and epileptic rats in the chronic phase of spontaneous seizures, and between vehicle- and drug-treated rats during epileptogenesis (prodromal phase) and at disease onset. The Area Under the Curve (AUC) and the cut off value (ng/ml) of total HMGB1 and corresponding isoforms concentration (sensitivity at 95% specificity) is reported for each experimental condition.

7.3.4 HMGB1 as a biomarker of epileptogenesis in the lithium+pilocarpine SE model

Since the changes in blood HMGB1 were detected in rats before the onset of the disease, we validated the potential role of HMGB1 for predicting epilepsy development using the lithium+pilocarpine SE model in which only the 70% of rats developed epilepsy.

The EEG and behavioral analysis of SE in pilocarpine-treated rats were concordant in showing that the animals were exposed to an inciting event of similar severity and duration (see Section 7.2). In accordance, SE-induced hippocampal neuronal cell loss, which is an objective measure of SE severity (Fujikawa, 1996), did not differ between *epileptic* and *non-epileptic* rats. Hippocampal cell loss in CA3 pyramidal cell layer and hilus was detected in epileptic rats (n=5) vs sham controls (not exposed to SE, n=7): number of cells, CA3, *Sham*, 546 ± 54; *Epileptic*, 454.5 ± 13 ($p<0.05$); Hilus, *Sham*, 93.9 ± 7.1; *Epileptic*, 38.9 ± 2.9 ($p<0.01$). Non epileptic rats had a similar pattern and extent of cell loss as epileptic rats (CA3, 443.7 ± 10.6; Hilus, 37.9 ± 3.3). Finally, the rats were classified as *epileptic* or *non-epileptic* based on two objective measures: the presence or absence of EEG seizures at 7 months post-SE and the hippocampal ADT (see Section 7.2).

Only those rats who developed epilepsy had increased blood levels of both total HMGB1 and its isoforms; these changes occurred before the onset of the disease and were maintained during disease development (Figure 7.6A). Non-epileptic rats were similar to controls at all time points post-SE (Figure 7.6A). The inactive sulfonyl HMGB1 isoform was not detected in any experimental condition. Moreover, *post-mortem* brain analysis showed that only epileptic rats displayed increased HMGB1 expression in the hippocampus at 7.5 months post-SE with concurrent nucleus-to-cytoplasm translocation in glial cells while non-epileptic rats were similar to controls (Figure 7.6B). The blood changes in HMGB1 did not merely reflect reactive gliosis since post-mortem GFAP-immunostaining was similar in the hippocampus of rats with

epilepsy (showing elevated blood HMGB1) or without epilepsy (with no elevation of blood HMGB1) as assessed in rats 7.5 months post-SE (Figure 7.6C).

ROC analysis showed perfect discrimination between epileptic and non-epileptic rats (AUC=1) with both total HMGB1 and its isoforms either measured before the onset of spontaneous seizures (prodromal phase), or at the onset of the disease, or in the chronic epilepsy phase (Table 7.2).

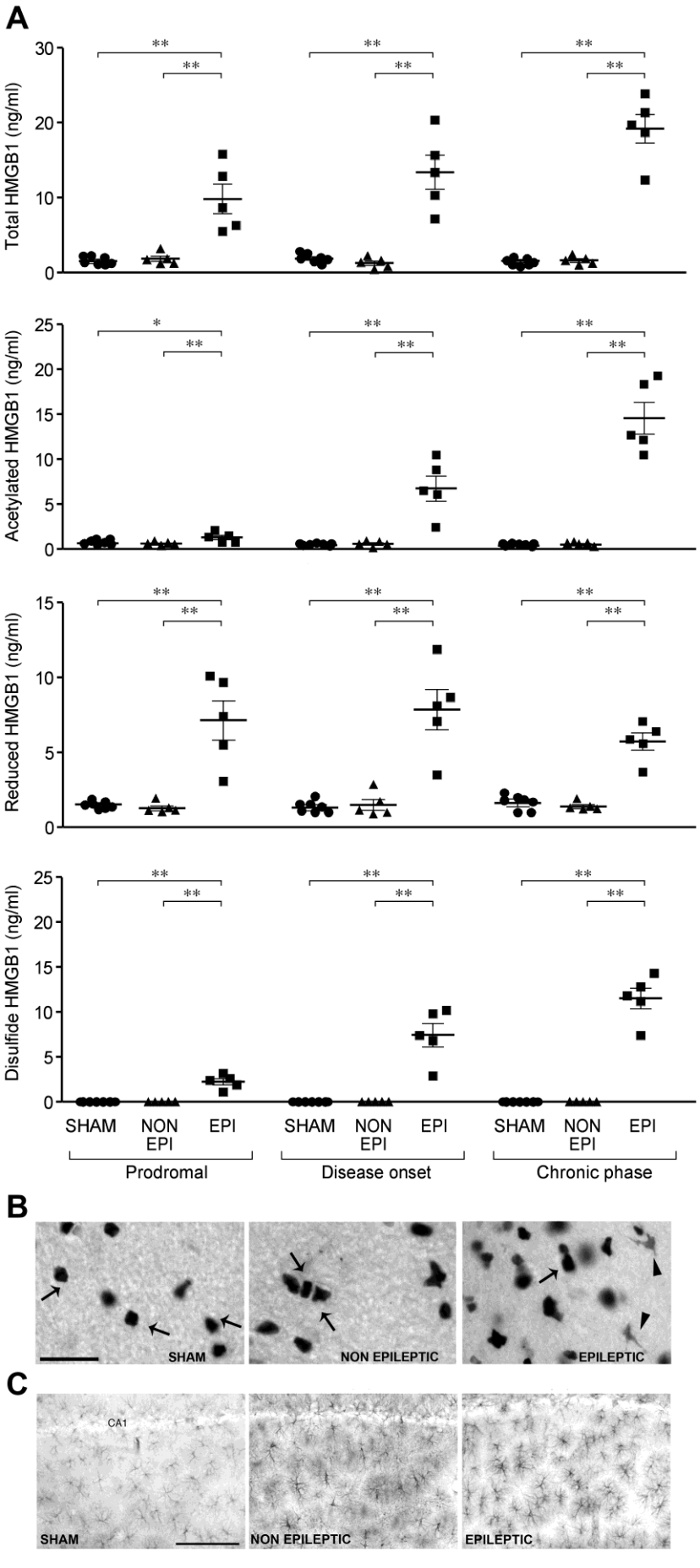

A
Total HMGB1 (ng/ml)
Acetylated HMGB1 (ng/ml)
Reduced HMGB1 (ng/ml)
Disulfide HMGB1 (ng/ml)
SHAM
NON EPI
EPI
Prodromal
Disease onset
Chronic phase
B
SHAM
NON EPILEPTIC
EPILEPTIC
C
CA1
SHAM
NON EPILEPTIC
EPILEPTIC

Figure 7.6. Early prediction of epilepsy development by monitoring blood HMGB1 level in lithium+pilocarpine SE-exposed rats. *Panel (A)*: Longitudinal analysis of total HMGB1 and acetylated, reduced and disulfide isoform levels in blood at representative time points of disease development. Rats are the same reported in Panel a. Blood was drawn at 23 days (epileptogenic phase prodromal to epilepsy onset), 73 days (encompassing the time of disease onset in 70% of rats) and 7.5 months (chronic epilepsy phase). Individual rat values are reported as dot plots; n=7 SHAM; n=5 EPILEPTIC (EPI); n=5 NON EPILEPTIC (NON EPI) rats. *p<0.05; **p<0.01 by Kruskal-Wallis test. Total HMGB1, acetylated and disulfide isoform levels in the chronic phase (EPI) are significantly different vs corresponding levels in prodromal phase (p<0.01 by repeated measures one-way ANOVA). *Panel (B)*: Representative immunohistochemical pictures of CA1 stratum radiatum depicting HMGB1 staining in control (SHAM, n=7) and in SE rats with (EPI, n=5 out of 12) or without (NON EPI, n=5) spontaneous seizures. Brains were harvested 7.5 months post-SE. Arrows show nuclear staining of HMGB1 in vehicle and non-epileptic rats; arrowheads show cytoplasmic HMGB1 staining in glia in epileptic rats. Scale bar: 15 μm. *Panel (C)*: Representative photomicrographs showing GFAP-immunoreactive astrocytes in the CA1 region. Epileptic and non epileptic rats (same rats as in panel B) show similar GFAP immunostaining in activated astrocytes exhibiting hypertrophic cell body, long and thick processes. Control rats show GFAP immunoreactivity in stellate-shaped astrocytes with thin processes denoting their resting state. Scale bar: 50 μm.

Table 7.2. *Receiver Operating Condition (ROC) analysis in pilocarpine SE-exposed rats.*

	Epileptic vs Non epileptic *Prodromal phase*				Epileptic vs Non epileptic *Disease onset*				Epileptic vs Non epileptic *Chronic phase*			
	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)	AUC	p value	Sensitivity at 95% specificity	Cut off (ng/ml)
Total HMGB1	1	0.0143	1 (0.4-1)	4.5	1	0.009	1 (0.5-1)	5.8	1	0.009	1 (0.5-1)	9.4
Ac-HMGB1	1	0.0143	1 (0.4-1)	0.8	1	0.009	1 (0.5-1)	1.4	1	0.009	1 (0.5-1)	6.2
Reduced HMGB1	1	0.0143	1 (0.4-1)	3.1	1	0.009	1 (0.5-1)	3.4	1	0.009	1 (0.5-1)	3.5
Disulfide HMGB1	1	0.0143	1 (0.4-1)	0.7	1	0.009	1 (0.5-1)	2.2	1	0.009	1 (0.5-1)	4.8

ROC analysis shows that blood HMGB1 level discriminates between epileptic and non-epileptic rats during disease development. The Area Under the Curve (AUC) and the cut off value (ng/ml) of total HMGB1 and corresponding isoforms concentration (sensitivity at 95% specificity) is reported for each experimental condition.

7.4 DISCUSSION

Electrically-induced SE in adult rodents increased non-acetylated and reduced HMGB1 isoforms in blood within hours. This is consistent with passive release of HMGB1 from injured neuronal and endothelial cells (Maroso et al., 2010), which is known to occur in the hippocampus after SE (Dingledine et al., 2014; Gorter et al., 2015). Following a different pilocarpine-induced epileptogenic insult, there was also an early increase in non-acetylated and reduced HMGB1 in the blood of rats that developed epilepsy, but not in those that did not, despite similar neuronal cell loss. This evidence suggests that the passive release of HMGB1 following SE does not only reflect cell death, but may also be a marker of the overall state of cell stress in neurons, glia and BBB endothelium induced by the epileptogenic insult.

Immunohistochemical data demonstrated that HMGB1 expression is predominantly increased in glial cells and endothelial cells of the BBB and translocates from nucleus-to-cytoplasm before the onset of spontaneous seizures. The pathologic isoform, i.e. disulfide HMGB1, and the acetylated isoform which translocates from nucleus-to-cytoplasm and can be released in the extracellular space, are both increased in the brain during early epileptogenesis as assessed by LC/MS-MS. This phenomenon is consistent with active release of HMGB1 from activated glial cells, as supported by immunohistochemistry. Notably, the brain changes in both acetylated and disulfide isoforms preceded the respective blood increases supporting either HMGB1 brain-to-blood leakage due to BBB damage (Gorter et al., 2015), or csf-to-blood diffusion (Hladky and Barrand, 2014). We cannot exclude, however, that leukocytes contribute to blood HMGB1 changes. Both the HPA axis and the vagal nerve can mediate signal of the inflammatory state in the brain to the blood, and this phenomenon could promote the release of HMGB1 from circulating leukocytes. It should be noted, however, that these brain-to-blood signals usually result in peripheral anti-inflammatory (rather than pro-inflammatory) responses (Gaillard, 2001).

Levels of HMGB1 and disulfide isoform after disease onset are increased progressively in animals exposed to electrical SE and only in rats developing epilepsy after SE induced by pilocarpine. Taken together, these results support the role for HMGB1 in the disease pathogenesis and indicate that its brain and blood changes are not solely a consequence of ongoing seizure activity. Indeed, HGMB1 and its isoforms increase during the early epileptogenesis phase, when no seizures occur, and before disease onset in two different experimental models. Secondly, there is no correlation between the number of seizures and the level of total HMGB1 in the chronic phase of epilepsy.

Moreover, the blood changes in HMGB1 did not merely reflect reactive gliosis since post-mortem GFAP-immunostaining was similar in the hippocampus of rats with epilepsy (showing elevated blood HMGB1) or without epilepsy (with no elevation of blood HMGB1) as assessed in rats 7.5 months post-SE.

While reduced HMGB1 has chemoattractant properties for macrophage/microglia (Yang et al., 2005, 2012), disulfide HMGB1 induces pro-inflammatory cytokine release from immune cells by activating TLR4 (Yang et al., 2005, 2012), and contributes to hyperexcitability and seizures in animal models by modulating Ca^{2+} permeability of NR2B-NMDAR (Balosso et al., 2014; Iori et al., 2013; Maroso et al., 2010). Additionally, TLR4 has been implicated in epileptogenesis (Iori et al., 2013). Therefore, the release of disulfide HMGB1 in the brain following SE may contribute to epileptogenesis and the consequent onset of spontaneous seizures. Accordingly, disulfide HMGB1 was generated in the rat brain before the onset of spontaneous seizures. Moreover, only rats who developed epilepsy, but not those without disease or controls, expressed this isoform in blood. The persistence of the pathological disulfide isoform in animals who developed epilepsy probably reflects inefficient resolution of neuroinflammation (De Simoni et al., 2000; Marcon et al., 2009).

The combined anti-inflammatory treatment aimed at blocking the broad inflammatory cascade originating from IL-1R1/TLR4 signaling prevented the increase in total, disulfide, acetylated and reduced HMGB1 levels in peripheral blood from disease onset. The suppression of HMGB1 and

its isoforms in blood at disease onset and during the chronic epilepsy phase *vis-à-vis* with the absence of a complete block of seizures in treated rats suggests that the anti-inflammatory treatment is indeed effective in reducing neuroinflammation but it was not sufficient to resolve other concomitant mechanisms involved in the pathogenesis of seizures (as described in Section 1.2.3). This experiment indicates that HMGB1 may be used as a non-invasive biomarker of response to anti-inflammatory treatments with disease-modifying effect.

Furthermore, since disulfide HMGB1 seems to be the isoform promoting seizure generation (Balosso et al., 2014), it would seem opportune to develop isoform-specific antagonists which are still lacking (Okuma et al., 2014; Yang et al., 2002).

The data presented in this study strongly support further investigations into the utility of total HMGB1 and its isoforms to act as biomarkers for epileptogenesis and predictors of therapeutic effects of new anti-inflammatory agents.

CHAPTER 8 - GENERAL DISCUSSION

The therapeutic control or prevention of pharmacoresistant epilepsies represents an unmet clinical need which is addressed in the field of experimental and clinical research. The next generation of therapies for the treatment of epilepsy should target the mechanisms intimately involved in the process of epileptogenesis with the final aim to prevent or positively modify the course of the disease. The identification of the proper targets and the optimal time window for intervention is crucial for the therapeutic success of anti-epileptogenesis or disease-modifying therapies. One critical step is to move from proof-of-concept anti-epileptogenesis studies in animal models to validation in preclinical trials, and eventually to clinical translation.

Epileptogenic insults, both in experimental models and in patients, determine a condition of neuroinflammation, characterized by the synthesis and release from innate immunity cells of pro-inflammatory molecules with neuromodulatory properties, among which IL-1β and TNF-α, and danger signals such as HMGB1 (Aronica et al., 2005; Boer et al., 2008; Ravizza and Vezzani, 2006; Ravizza et al., 2011). These molecules play a pathogenic role during epileptogenesis in animal models (Vezzani et al., 2013). Experimental evidence and clinical observations indicate that the inflammatory response to epileptogenic events is not efficiently controlled by endogenous anti-inflammatory and homeostatic mechanisms (Aronica et al., 2007; De Simoni et al., 2000; Pernhorst et al., 2013; Ravizza et al., 2006b; Sun et al., 2016). This means that lack of efficient resolution mechanisms may be a crucial factor leading to persistent and excessive neuroinflammation thereby resulting in brain dysfunction. In support, pharmacological or genetic interventions aimed at increasing the levels of an endogenous anti-inflammatory molecules, such as IL-1R antagonist, or reducing the extent of the neuroinflammatory response to injuries by blocking the inflammasome or the cytokine receptors, drastically reduced the frequency of seizures and in some instances delayed their onset in experimental models (Maroso et al., 2011b; Ravizza and Vezzani, 2006; Vezzani et al., 2000, 2011c). Among the endogenous molecules with anti-inflammatory and "pro-resolving" properties, there are specialized pro-resolving mediators (SPMs), namely, lipids derived from arachidonic acid and n-

3 polyunsaturated fatty acids (e.g. LXA_4, RvD1, PD1), and peptides (e.g. Annexin A1). SPMs binding to their specific receptors (such as ALXR and ChemR23) drives the active resolution of inflammation, by reducing the production of pro-inflammatory cytokines, the recruitment of immune cells and increasing the synthesis of anti-inflammatory molecules.

The hypothesis underlying the research projects described in this thesis is based on the consideration that neuroinflammation following an epileptogenic insult is not efficiently controlled by specialized resolution mechanisms. If neuroinflammation persists and becomes chronic, it may contribute to seizure onset and recurrence, and to the concomitant neuropathology and neurological deficits. Therefore, in this study key pro-resolving mechanisms were characterized during epileptogenesis. Moreover, two pharmacological interventions aimed at preventing or limiting the pathological consequences of neuroinflammation were performed. One approach potentiated the resolution of inflammation and the other blocked specific pro-inflammatory signalings. Finally, a pro-inflammatory molecule such as HMGB1 was studied as a potential biomarker of epileptogenesis.

In the first part of this thesis, a mouse model of epileptogenesis induced by kainic acid was used to study the time of induction of resolution molecules versus major neuroinflammatory cytokines in the disease phase preceding the onset of spontaneous seizures. The analyses were performed in the hippocampus which is involved in the recurrence and generalization of seizures. Moreover, this brain region –together with cortical areas- plays a major role in cognitive deficits developing in epileptic mice and in humans with pharmacoresistant seizures.

The major findings showed that neuroinflammation was rapidly evoked and persisted for days after the primary epileptogenic insult while the activation of resolution mechanisms was significantly delayed and more transient. In fact, the levels of pro-resolving receptors, ALXR and ChemR23, the main biosynthetic enzymes LOX5 and LOX15, and the pro-resolving peptide Annexin A1 were all upregulated later than the pro-inflammatory counterpart. Moreover, lipidomic analysis showed an alteration of the synthesis and metabolism of lipid mediators

during epileptogenesis. Specifically, an up-regulation of DHA- and n-3DPA-derived neuroprotectins and EPA-derived E-series resolvins was concomitant with a down-regulation of AA-derived lipoxins and of DHA- and n-3DPA-derived D-series resolvins. In particular, PD1 derived from n-3DPA ($PD1_{n\text{-}3DPA}$) was the one mostly up-regulated during epileptogenesis suggesting that this lipid mediator might be one of the key player in the resolution of neuroinflammation. Moreover, supporting findings showed therapeutic effects of an analogue of $PD1_{n\text{-}3DPA}$ on spontaneous seizures. Indeed, PD1 derived from DHA reduced hippocampal hyperexcitability following electrical stimulation and spontaneous seizure frequency in a mouse model of epileptogenesis induced by pilocarpine administration (Musto et al., 2011, 2015). Based on this evidence, two approaches were designed in order to anticipate and strengthen resolution mechanisms during epileptogenesis. The pharmacological treatments were transiently administered during the epileptogenesis phase before disease onset starting 1 h after the epileptogenic insult. The first approach was aimed at boosting ALXR receptor activation using two of its agonists: a stable analogue of LXA_4 or a peptide derived from the N-terminal portion of Annexin A1. The second approach was aimed at increasing the level of $PD1_{n\text{-}3DPA}$ using a synthetic stable analogue ($PD1_{n\text{-}3DPA}ME$). These interventions were supported by experimental data showing that the administration of analogues of LXA_4 and Annexin A1 reduced neuroinfiammation after neurotrauma, in neuropathic pain, ischemia and AD (Gavins et al., 2007; Hawkins et al., 2014; Luo et al., 2013; Martini et al., 2016; Medeiros et al., 2013; Svensson et al., 2007; Wu et al., 2012a; Ye et al., 2010). However, our interventions for potentiating ALXR activation did not affect IL-1β levels during epileptogenesis, thus indicating that they were not effective in reducing neuroinflammation. One possible explanation for the lack of anti-inflammatory effects of these molecules may rely upon the evidence that ALXR can respond also to ligands, such as Amyloid β, serum amyloid protein A, IL-8 and CCL23 that may promote the inflammatory response (Le et al., 2001; Waechter et al., 2012). Amyloid β-protein precursor (APP) is increased in temporal lobe epilepsy (Sheng et al., 1994) and after electroconvulsive shock in mice (Jang et al., 2016). Moreover, neuronal expression of APP is

associated with heightened IL-1 immunoreactivity in human temporal lobe epilepsy (Sheng et al., 1994). It is possible that SE-induced release of IL-1 from glial cells leads to APP production in neurons and stimulation of ALXR during the initial phases of epileptogenesis. IL-8 concentration in serum and csf were increased rapidly after seizures in patients with refractory epilepsy, febrile SE, severe head injury and neonatal seizures induced by hypoxic-ischaemic encephalopathy and remained up-regulated for 4 days thereafter (Billiau et al., 2007; Gallentine et al., 2017; Whalen et al., 2000; Youn et al., 2012). Finally, levels of CCL23 were up-regulated in RE affected children (Owens et al., 2013). Alternatively, the lack of effect of the two treatments might be explained by a sub-optimal treatment protocol due to their limited solubility which prevented the possibility of injecting icv higher concentrations of the molecules.

Differently, $PD1_{n\text{-}3DPA}ME$ significantly decreased neuroinflammation during epileptogenesis, improved post-injury weight recovery in mice, rescued the cognitive deficits and reduced spontaneous seizure frequency and duration in the early stages of the disease. In accordance with our findings, treatment with PD1 derived from DHA reduced astrocyte and microglia activation and downregulated cytokine mediated activation of pro-inflammatory signaling in an experimental model of AD and protected human retinal cells from oxidative stress (Mukherjee et al., 2004; Zhao et al., 2011).

Further studies will assess the effect of $PD1_{n\text{-}3DPA}ME$ on disease progression, evaluating spontaneous seizure frequency also in the late stages of the disease. Indeed, data from our laboratory demonstrated that anti-inflammatory and anti-oxidant drugs reduced the progression of spontaneous seizures, thus showing disease-modifying effect (Iori et al., 2017b; Pauletti et al., 2017).

In conclusion, these results support the idea that delayed resolution may favor the establishment of persisting neuroinflammation. Anticipating and boosting the endogenous pro-resolving response, with stable analogues of lipid mediators such as $PD1_{n\text{-}3DPA}$, may

represent a rational approach to reduce or prevent the pathological consequences of neuroinflammation.

Specific mimetic drugs of endogenous pro-resolving mediators might be used as adjunctive therapy together with the anti-seizures drugs in patients with drug-resistant seizures or in patients with first presentation of seizures or exposed to epileptogenic injuries.

Another approach to counteract inflammation during epileptogenesis is to use specific anti-inflammatory treatments. In particular, our laboratory has demonstrated that IL-1β/IL-1R1 and HMGB1/TLR4 axes are activated during seizures and after epileptogenic injuries in humans and in the corresponding animal models (Vezzani et al., 2011b). Targeting the single components of this pathway drastically reduced induced or spontaneous seizures (Iori et al., 2013; Maroso et al., 2010; Vezzani et al., 2000). Moreover, targeting IL-1-β/IL-1R1 axis was not sufficient to prevent the activation of TLR4 signaling which *per se* play a role in epileptogenesis (Vezzani et al., 2013). These findings raised the hypothesis that a combination of drugs, targeting IL-1R1 and TLR4, was required to attain an efficient inhibition of epileptogenesis. The drug combination used in this research project included two anti-inflammatory molecules: Anakinra, the human recombinant form of IL-1R1 antagonist, and BoxA, a fragment of HMGB1 with antagonist activity on TLR4. In addition, Ifenprodil was used to block NR2B-NMDARs which represent one molecular target of IL-1-β/IL-1R1 and HMGB1/TLR4 signaling mediating excitotoxicity (Balosso et al., 2008, 2014). The combined treatment was transiently administered for 1 week during epileptogenesis starting 1 h after the epileptogenic insult in adult rats. This treatment protocol did not affect SE and the associated neuropathology (Noe et al., 2013; Pauletti et al., 2017) while it prevented the progression of the disease. Treated rats did not show the 4-fold progression in spontaneous seizures displayed by vehicle controls. In accordance with our data, there is evidence that anti-inflammatory drug combinations using anakinra together with a COX-2 inhibitor or VX-765 together with a TLR4 antagonist, or an epigenetic approach using miRNA146a which inhibits both the IL-1R1 and TLR4 signaling,

prevented disease progression and reduced chronic seizure recurrence (Iori et al., 2017b; Quinn and O'Neill, 2011; Vezzani et al., 2015).

The last part of this research project concerned the characterization and validation of circulating isoforms of HMGB1 as mechanistic biomarkers of epileptogenesis in animal models. The findings showed that HMGB1 isoforms can be reliably measured in blood and they reflect the respective brain modifications. Early after the epileptogenic injury, the non acetylated and reduced isoforms measured in blood reflect passive release of HMGB1 from injured or stressed neuronal, glial and endothelial cells (Dingledine et al., 2014; Gorter et al., 2015; Maroso et al., 2010; Yang et al., 2012). At later time points of epileptogenesis, notably before the onset of spontaneous seizures, and during disease development both acetylated and disulfide isoforms progressively increased and accounted for the majority of total HMGB1 blood levels. This phenomenon is consistent with active release of HMGB1 from activated glial cells, and is predictive of epilepsy onset in animal models with high fidelity. Blood HMGB1 may reflect brain-to-blood leakage due to BBB damage (Gorter et al., 2015), or csf-to-blood diffusion (Hladky and Barrand, 2014), although we cannot exclude a contribution of peripheral leukocytes to HMGB1 blood changes (Lu et al., 2013).

The data therefore support a role for HMGB1 as a mechanistic biomarker of epileptogenesis. Accordingly, disulfide HMGB1 binding to TLR4 contributes to hyperexcitability and seizures in animal models by modulating Ca^{2+} permeability of NR2B-NMDAR (Balosso et al., 2014; Iori et al., 2013; Maroso et al., 2010). The release of disulfide HMGB1 in the brain following SE may contribute to epileptogenesis and the consequent onset of spontaneous seizures. The persistence of the pathological disulfide isoform in animals who developed epilepsy probably reflects inefficient resolution of neuroinflammation (De Simoni et al., 2000; Marcon et al., 2009).

In order to determine the relevance of animal findings for human epilepsy, our laboratory in collaboration with the Univeristy of Liverpool undertook bridging studies in patients with drug-resistant and newly-diagnosed epilepsy. Patients with long-standing, drug-resistant epilepsy

had significantly higher total HMGB1 levels in blood compared to patients with well-controlled epilepsy or healthy controls. Only patients with drug-resistant seizures expressed the acetylated, actively secreted form of HMGB1. This is consistent with a previous study showing the cytoplasmic translocation of HMGB1 in neurons and glia in surgically resected brain tissue from drug-resistant patients (Maroso et al., 2010; Zurolo et al., 2011).

Consistent with our findings in animal models, a pilot study in newly-diagnosed patients revealed the presence of disulfide HMGB1 not normally seen in the sera of healthy individuals or patients with longstanding, well-controlled epilepsy. Persistent elevation of these pathological isoform following epilepsy diagnosis was associated with subsequent seizures, whereas disappearance of this isoform, despite elevated total HMGB1 levels, was associated with a reduced likelihood of subsequent seizures. These data suggest that disulfide HMGB1 that is elevated early in the disease course may be a useful prognostic and predictive biomarker of ongoing seizure activity despite anti-seizure therapy. Moreover, the disulfide HMGB1 isoform may identify patients with a pathologic neuroinflammatory *milieu.* A limitation of our findings is the small number of patients studied so far, thus, a prospective, longitudinal study in newly-diagnosed patients with epilepsy is required to validate these findings. Moreover, our hypothesis can be tested in prospective human clinical studies, such as in post-stroke or post-traumatic epilepsy, by examining the prognostic value of total HMGB1 and its isoforms before the first presentation of seizures.

Our data both in animals and humans indicate that the changes in blood HMGB1 are not solely reflecting ongoing seizure activity. In the animal models of epilepsy, such changes occurred before the onset of spontaneous seizures. Moreover, HMGB1 blood levels did not correlate with the number of seizures in the animals or patients with epilepsy. Lastly, should HMGB1 purely reflect seizure activity then one would expect those patients with the most recent seizures to express higher levels of the biomarker. We did not find this to be case in our studies.

The current prognostic models for seizure recurrence following a first seizure, and for seizure remission following a diagnosis of epilepsy, are based on clinical factors including age, seizure type, EEG and MRI [MESS and SANAD models (Bonnett et al., 2012; Kim et al., 2006)], but lack precision. These models could be much improved by the addition of circulating biomarkers such as HMGB1 and its isoforms.

Finally, the evidence obtained by measuring blood HMGB1 in rats under anti-inflammatory treatment supports that blood HMGB1 isoforms may predict the therapeutic response to such drugs.

CHAPTER 9 – SUMMARY AND CONCLUSIONS

The research project described in this thesis has provided supporting evidence to the concept that the resolution mechanisms of neuroinflammation are impaired during epileptogenesis and that incrementing the resolution response with specific pro-resolving molecules may improve pathologic outcomes. Moreover, pharmacological targeting of neuroinflammation early post-injury with drugs some of which are already used clinically prevented the progression of the disease and drastically reduced spontaneous seizures.

A major challenge is the translation of these experimental results into clinically effective human therapies. Indeed, clinical trial designs for novel therapeutics would greatly benefit from non-invasive biomarkers such as HMGB1 that allow early identification of patients at high risk of developing the disorder, and those patients who might preferentially respond to disease-modifying treatments.

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